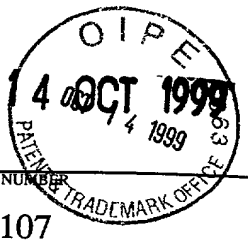
412 Rec'd PCT/PTO



FORM PTO-1390 (Modified) (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 082584/0107

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5): 09/402936

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/07388	April 14, 1998	April 14, 1997

TITLE OF INVENTION: RECOMBINANT bHLH-PAS/JHR POLYPEPTIDE AND ITS USE TO SCREEN POTENTIAL INSECTICIDES

APPLICANT(S) FOR DO/EO/US: **Thomas G. WILSON and Julia N. HEINRICH**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371 (c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☒ A copy of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/402936	PCT/US98/07388	082584/0107

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17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5):		
Search Report has been prepared by the EPO or JPO **$840.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) **$670.00**		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2) **$760.00**		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$970.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) **$96.00**	96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e))	$	

Claims	Number Filed		Number Extra	Rate		
Total Claims	**51**	-20 =	**31**	X **$18.00**	$ **558.00**	
Independent Claims	**19**	-3 =	**16**	X **$78.00**	$ **1,248.00**	
Multiple dependent claim(s) (if applicable)				+ **$260.00**	$ **0.00**	
TOTAL OF ABOVE CALCULATIONS =					$ **1,902.00**	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).					$	
SUBTOTAL =					$ **1,902.00**	
Processing fee of **$130.00** for furnishing English translation later the ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f).					$	
TOTAL NATIONAL FEE =					$ **1,902.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					$	
TOTAL FEES ENCLOSED =					$ **1,902.00**	
					Amount to be: refunded $	
					charged $	

a. ☒ A check in the amount of **$1,902.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 19-0741 in the amount of $_____ to the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0741. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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31,298
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AH34

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RECOMBINANT bHLH-PAS/JHR POLYPEPTIDE AND ITS USE TO SCREEN POTENTIAL INSECTICIDES

BACKGROUND OF THE INVENTION

The present invention relates to a cloned "basic helix loop helix -PER-ARNT-AhR-SIM" (bHLH-PAS) protein that is a juvenile hormone receptor (JHR), bHLH-PAS/JHR. In particular, this invention is directed to a bHLH-PAS/JHR gene isolated from *Drosophila*, termed the *methoprene-tolerant (met)* gene (*Met*-JHR). The present invention also is directed to *in vitro* and *in vivo* methods for screening insecticides using recombinant bHLH-PAS/JHRs. The present invention is further directed to methods for isolating polynucleotides encoding bHLH-PAS/JHRs from various insect species.

Worldwide insect damage to food and fiber costs billions of dollars annually. Although chemical insecticides are still the primary means of insect control, the use of chemicals has several drawbacks including high cost of discovery, potential environmental damage, and negative public opinion. One promising group of insecticides consists of analogues of insect hormones, such as juvenile hormone. Since vertebrates do not make juvenile hormone (JH), insecticides targeted to the JH system are highly toxic to certain insects, and have shown an extraordinary degree of environmental safety.

Juvenile hormones comprise a family of hormones that are secreted by the corpus allatum, and that play a role in a variety of critical functions in insects, including development, reproduction, and morphological differentiation. Riddiford, "Hormone Action at the

Cellular Level," in COMPREHENSIVE INSECT PHYSIOLOGY, BIOCHEMISTRY AND PHARMACOLOGY, VOLUME 8, Kerkut *et al.* (eds.), pages 37-84 (Pergamon Press 1985); Nijhout *et al.*, *Q. Rev. Biol.* 57:109 (1982). These hormones affect development in some insects by maintaining the larval stage and inhibiting metamorphosis. In adult insects, JH is involved in the regulation of reproductive physiology. Koeppe *et al.*, "The Role of Juvenile Hormone in Reproduction," in COMPREHENSIVE INSECT PHYSIOLOGY, BIOCHEMISTRY AND PHARMACOLOGY, VOLUME 8, Kerkut *et al.* (eds.), pages 165-203 (Pergamon Press 1985).

The action of JH is mediated by at least several types of JH binding proteins: a hemolymph carrier protein, a cell membrane bound receptor, and an intracellular receptor. The transport of JH to target tissues is believed to be accomplished by proteins in the hemolymph which bind with the hormone. Hammock *et al.*, *Pestic. Biochem. Physiol.* 7:517 (1977); Goodman *et al.*, "Juvenile Hormone Cellular and Hemolymph Binding Proteins," in COMPREHENSIVE INSECT PHYSIOLOGY, BIOCHEMISTRY AND PHARMACOLOGY, VOLUME 7, Kerkut *et al.* (eds.), pages 491-510 (Pergamon Press 1985). These JH binding proteins are thought to play roles both in the transport of JH and in the protection of JH from hemolymph esterases. Goodman *et al.*, *Am. Zool.* 14:1289 1974; Kramer *et al.*, *J. Biol. Chem.* 251:4979 (1974). Membrane bound receptors are known to bind ligand extracellularly and transmit a signal intracellularly. Wyatt *et al.* *Adv. Insect Physiol.* 26:1 (1996).

Cytosolic proteins that bind JH have been identified in numerous JH target tissues from a variety of insects. Van Mellaert *et al.*, *Insect Biochem.* 15:655 (1985); Klages *et al.*, *Nature* 286:282 (1980); Engelmann *et al.*, *Insect Biochem.* 17:1045 (1987); Wisniewski *et al.*, *FEBS Lett.* 171:127 (1984). One of the inventors, Thomas G. Wilson, directed a research team that identified a

cytosolic juvenile hormone-binding protein in *Drosophila melanogaster* that is characterized by saturable, high-affinity binding specific for JH III. Shemshedini *et al.*, *J. Biol. Chem.* *265*:1913 (1990). Shemshedini *et al.* also demonstrated for the first time in any insect a correlation between the binding of JH to the cytosolic protein and a biological response to the hormone. Interference with the binding of JH to cognate intracellular receptors, therefore, would inhibit physiological functions dependent upon the hormone.

Until recently, novel insecticides that interfere with JH action were primarily discovered by an almost random testing of thousands of chemical compounds for efficacy against insects. This bioassay approach is slow and expensive since a group of test insects would have to be treated with various doses of each test compound, and, typically, finding compounds that are effective is exceedingly rare.

Accordingly, a need exists for an efficient method for testing insecticides targeted for the JH system.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide *in vitro* and *in vivo* assays for screening potential insecticides that are JH analogs and JH antagonists.

Another object of this invention is to provide methods for cloning bHLH-PAS/JHR genes from various insect species.

These and other objects are achieved, in accordance with one embodiment of the present invention by the provision of an isolated polynucleotide that comprises an insect bHLH-PAS/JHR gene, the *Met*-JHR gene. A "polynucleotide" includes DNA, RNA, mRNA, and cDNA

molecules. A genomic polynucleotide comprising the *Met* gene is the St-H fragment in Figure 1. This fragment is 6.234 Kb, and its sequence is shown in Figure 2 (SEQ ID NO:1). Within this 6.234 Kb segment, there is a DNA sequence of 3.011 Kb, which includes an open reading frame that is divided by one intron of 69 nucleotides (bases 1520 to 1588). This 3.011 Kb sequence is the genomic *Met*-JHR DNA sequence. Figure 3 (SEQ ID NO:2).

The *Met*-JHR open reading frame lacking the intron codes for a protein of 716 amino acids and a having a molecular weight of about 78,720 daltons.

The nucleotide sequences of the genomic and cDNA *Met*-JHR differ, reflecting polymorphism. In Figure 3, SEQ ID NO:3 represents a *Met*-JHR cDNA sequence, which begins at nucleotide 4 of the genomic sequence. There is one "polymorphic" difference between the genomic and cDNA nucleotide sequences that results in a change at the amino acid level. The nucleotide at position 1043 (genomic)/1039 (cDNA) may be C or T, which results in different deduced amino acids, R and W, respectively.

In the sequence of the genomic DNA, there is one ambiguity that results in different deduced amino acids. Base number 875 in the genomic DNA is designated "R," which signifies that the nucleotide may be the purine C or G. This results in two possible corresponding deduced amino acid sequences, G (Gly) or R (Arg) respectively. In the sequence of the cDNA, there is one ambiguity that results in a different deduced amino acids. Base number 526 in the genomic DNA is designated "M," which signifies that the nucleotide may be the purine A or C. This results in two possible corresponding deduced amino acid sequences, T (Thr) or P (Pro) respectively.

As used herein, the term "juvenile hormone receptor" (JHR) is used to mean a polypeptide that is involved in binding JHIII. As used herein, a polypeptide that is "involved in binding" JHIII includes a

polypeptide that directly binds JHIII, a polypeptide that is a partner to a polypeptide that directly binds JHIII, and a polypeptide that is a partner to a complex of polypeptides that bind JHIII. One or more of these polypeptides may be required for binding JHIII. The skilled artisan will recognize that heterodimeric receptors are known in the art, and that both polypeptide that form the heterodimer are required for hormone binding and activity in the target cell. For example, the *ultraspiracle* polypeptide is partner to the ecdysone receptor, which together bind the hormone ecdysone and mediate ecdysone activation of gene transcription. Yao, *et al. Cell* 71:63 (1992).

A multicomponent complex between bHLH-PAS polypeptide and steroid receptors has been documented. For example, a bHLH-PAS polypeptide that functions a co-activator during ligand induction of estrogen steroid receptor is amplified in breast cancer-1 (AIBC or ACTR). Anzick *et al. Science*, 277:956 (1997); Chen *et al. Cell* 90:569 (1997). The JHR may involve a number of polypeptides that together form a ligand binding unit or functional signal transducing complex.

Thus, a suitable insect JHR gene encodes a polypeptide that directly binds to JHIII. Another suitable insect JHR gene encodes a polypeptide that is a heteromultimeric partner to a polypeptide that directly binds JHIII. A further suitable insect JHR gene encodes a polypeptide that forms a homomultimeric complex that binds JHIII. A suitable JHR is a bHLH-PAS/JHR polypeptide, *i.e.*, a bHLH-PAS polypeptide that is involved in binding juvenile hormone III. Such a bHLH-PAS/JHR polypeptide includes, but is not limited to, the *Met*-JHR polypeptide and the *Met*-JHR-*erecta* polypeptide.

As used herein, a "bHLH-PAS protein" is a member of a family of transcriptional activators known as the basic helix-loop-helix-Per-Arnt-*Sim* (bhlh-PAS) proteins.

These proteins share homology in two domains. The first domain is located at the N-terminus of the protein and comprises a region of basic amino acids followed by a region of approximately 50 conserved amino acids that form two amphipathic α helices that are joined by a variable loop: the basic domain and the helix loop helix are collectively referred to as bHLH.

The second domain is located immediately C-terminal to the bHLH domain and consists of approximately 300 amino acids termed PAS homology domain (for their original observation in the *Drosophila* midline development protein single-minded (sim) and the *Drosophila* circadian oscillator Period (per)). The PAS domain contains two copies of an approximately 50-amino acid degenerate repeat, referred to as the PAS A and PAS B repeats.

Additionally, some members of the bHLH-PAS family have at their C-termini a transcriptional activator domain, also called transactivation domain (TAD), which has been divided into three distinct classes corresponding to amino acid composition: rich in glutamines (Q-rich); rich in acidic amino acids (i.e., aspartate and glutamate); or with a high concentration of prolines, serines, and/or threonines (P/S/T).

The basic region of a bHLH-PAS protein is associated with DNA binding, and the HLH and PAS domains are associated with DNA binding and dimerization functions. The founding member of this family is the aryl hydrocarbon nuclear receptor translation (ARNT), so named because it was considered to translocate the ligand-bound aryl hydrocarbon receptor (AhR) to the nucleus. AhR is the only member in the bHLH-PAS family known to bind a ligand.

The ARNT receptor which is not bound to ligand is associated with two proteins, the 90 kDa heat shock protein and an unidentified 43 kDA protein, and ligand

binding is concomitant with dissociation of these proteins. Association with these proteins and binding of the ligands has been mapped to the same region, the middle third portion of the AhR protein, which includes the PAS B domain approximately in the middle.

The formation of a heterodimeric complex between the ligand-bound AhR and ARNT permits the complex to bind its enhancers, i.e., the dioxin or xenobiotic response elements (DRE or XRE, respectively) and induces transcription of specific genes.

Another family member, the hypoxia-inducible factor 1 alpha receptor (HIF-1α), which appears to sense low oxygen levels in the cell (hypoxia), also forms heterodimeric complexes with ARNT. In the presence of low oxygen, the HIF-1α/ARNT heterodimeric complex binds HIF-1α response elements (enhancers), and thereby induces gene transcription. *sim* also appears to heterodimerize with ARNT. The AhR/ARNT heterodimeric complex has been used as a model system to study the mechanism by which these family members transduce intracellular signals. Rowlands *et al. Critical Reviews in Toxicology*, 27: 109 (1997).

A suitable insect bHLH-PAS/JHR polynucleotide has a nucleotide sequence that encodes an amino acid sequence of SEQ ID NO:4 or SEQ ID NO:5. Other suitable bHLH-PAS/JHR polynucleotides comprise the nucleotide sequence of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:7. The present invention also contemplates host cells comprising such polynucleotides, and methods of using such host cells to produce bHLH-PAS/JHR.

Thus, the present invention provides an isolated polynucleotide that encodes a bHLH-PAS polypeptide that is involved in binding JHIII, also designated bHLH-PAS/JHR. The invention also includes a polynucleotide that encodes a bHLH-PAS/JHR polypeptide that directly binds juvenile hormone III, and a bHLH-PAS/JHR

polypeptide that directly binds juvenile hormone III as a monomer. The invention also includes a polynucleotide that encodes a bHLH-PAS/JHR polypeptide that directly binds juvenile hormone III as a homomultimer. The invention further includes an isolated polynucleotide encoding a bHLH-PAS/JHR polypeptide, wherein said polynucleotide encodes a polypeptide that is a heteromultimeric partner of a polypeptide that directly binds juvenile hormone III.

The invention includes an isolated polynucleotide encoding an insect bHLH-PAS/JHR polypeptide, wherein the insect is selected from the group consisting of *Coleoptera, Siphonoptera, Orthoptera, Thysanoptera, Lepidoptera, Hemiptera, and Diptera.* Members of *Diptera* may be selected from the group consisting of horn fly, fruit fly, screwworm fly, blow fly, mosquito, mediterranean fruit fly, biting midge, black fly, horse fly, deer fly, stable fly, leaf miner, housefly, bot fly, warble fly, tiger mosquito, swamp marsh mosquito, *Culex pipieus, Aedes aegypti,* and *Anopheles albopictus.*

The invention includes an isolated polynucleotide that encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:4 (Figure 4) and SEQ ID NO:5 (Figure 4). The invention further includes an isolated polynucleotide that comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3 (Figure 3). The invention also includes an isolated polynucleotide that comprises the nucleotide sequence selected from the group consisting of SEQ ID NO:6 and SEQ ID NO:7 (Figure 5).

The invention also includes an isolated polynucleotide which comprises the sequence of SEQ ID NO:1, an isolated polynucleotide which comprises the sequence of nucleotide 1 through nucleotide 1291 of SEQ ID NO:1, an isolated polynucleotide which comprises the sequence of nucleotide 1 through nucleotide 1513 of SEQ

ID NO:1, an isolated polynucleotide which comprises the sequence of nucleotide 3733 through nucleotide 6235 of SEQ ID NO:1, and an isolated polynucleotide which comprises the sequence of nucleotide 4302 through nucleotide 6235 of SEQ ID NO:1.

The invention also includes an isolated polynucleotide comprising the nucleotide sequence of the St-H fragment in vector pSt-H, which was deposited at the American Type Culture Collection, in Bethesda, Maryland, on November 13, 1997.

The invention further comprises an isolated polynucleotide comprising the nucleotide sequence of SEQ ID NO:7 (Figure 5).

The invention includes an isolated polynucleotide that encodes a bHLH-PAS/JHR polypeptide that is involved in binding JHIII, and that hybridizes under stringent conditions with a polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:6. The invention also includes an isolated polynucleotide that encodes a bHLH-PAS/JHR polypeptide that is involved in binding JHIII, and that hybridizes under stringent conditions with a polynucleotide having a nucleotide sequence of SEQ ID NO:7. The invention also includes an isolated polynucleotide that encodes a bHLH-PAS/JHR polypeptide that is involved in binding JHIII, and that hybridizes under stringent conditions with a polynucleotide that encodes a protein having the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5.

The invention also includes an isolated polynucleotide that encodes a bHLH-PAS/JHR polypeptide that is involved in binding JHIII and that hybridizes under stringent conditions with a riboprobe that is the reverse transcript of a polynucleotide having the sequence of nucleotide 1514 through 1845 of SEQ ID NO:1

(nucleotides 771 to 1102 of the *Met*-JHR open reading frame). The invention further includes an isolated polynucleotide that encodes a bHLH-PAS/JHR polypeptide that is involved in binding JHIII, and that hybridizes with a riboprobe that is the reverse transcript of a polynucleotide having the sequence of nucleotide 1514 through 1845 of SEQ ID NO:1 (nucleotides 771 to 1102 of the *Met*-JHR open reading frame), wherein said hybridization is carried out in 5X SSPE, 5X Denhardt's, 0.5% SDS, 50% formamide, and 100 μg/ml yeast tRNA for about 15 to about 17 hours at 68°C.

The invention includes an expression vector comprising an isolated polynucleotide encoding an insect bHLH-PAS/JHR polypeptide, and A cultured host cell comprising such an expression vector. The host cell is selected from the group consisting of bacterial cell, yeast cell, insect cell and mammalian cell.

The invention also includes a method of producing a polypeptide, said method comprising the steps of:

(a) culturing a host cell comprising an expression vector that comprises a bHLH-PAS/JHR gene, wherein said cultured host cell expresses said bHLH-PAS/JHR gene, and

(b) isolating said polypeptide from said cultured host cell.

The invention also includes an isolated polypeptide selected from the group consisting of:

(a) a conservative amino acid variant of SEQ ID NO:4,

(b) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:4,

(c) a polypeptide having an amino acid sequence of SEQ ID NO:4,

(d) a conservative amino acid variant of SEQ ID NO:5,

(e) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:5,

(f) a polypeptide having an amino acid sequence of SEQ ID NO:5, and

(g) a *Met*-JHR alternatively-spliced isoform.

The invention also includes the above-mentioned isolated polypeptides, wherein the conservative amino acid variant is a polypeptide having an amino acid sequence that differs from the amino acid sequence of SEQ ID NO:4 by containing at least one amino acid substitution selected from the group consisting of (1) the substitution of an alkyl amino acid for an alkyl amino acid in SEQ ID NO:4, (2) the substitution of an aromatic amino acid for an aromatic amino acid in SEQ ID NO:4, (3) the substitution of a sulfur-containing amino acid for a sulfur-containing amino acid in SEQ ID NO:4, (4) the substitution of a hydroxy-containing amino acid for a hydroxy-containing amino acid in SEQ ID NO:4, (5) the substitution of an acidic amino acid for an acidic amino acid in SEQ ID NO:4, (6) the substitution of a basic amino acid for a basic amino acid in SEQ ID NO:4, and (7) the substitution of a dibasic monocarboxylic amino acid for a dibasic monocarboxylic amino acid in SEQ ID NO:4.

The invention further includes the above-described isolated polypeptides, wherein the conservative amino acid variant is a polypeptide having an amino acid sequence that differs from the amino acid sequence of SEQ ID NO:5 by containing at least one amino acid substitution selected from the group consisting of (1) the substitution of an alkyl amino acid for an alkyl amino acid in SEQ ID NO:5, (2) the substitution of an aromatic amino acid for an aromatic amino acid in SEQ ID NO:5, (3) the substitution of a sulfur-containing amino acid for a sulfur-containing amino acid in SEQ ID NO:5, (4) the substitution of a hydroxy-containing amino acid

for a hydroxy-containing amino acid in SEQ ID NO:5, (5) the substitution of an acidic amino acid for an acidic amino acid in SEQ ID NO:5, (6) the substitution of a basic amino acid for a basic amino acid in SEQ ID NO:5, and (7) the substitution of a dibasic monocarboxylic amino acid for a dibasic monocarboxylic amino acid in SEQ ID NO:5.

The invention further includes a method for screening compounds that specifically bind with a bHLH-PAS/JHR polypeptide, comprising:

(a) incubating a test compound in a solution that comprises an isolated recombinant bHLH-PAS/JHR polypeptide, and

(b) detecting the binding of said test compound with said polypeptide.

The invention includes a method for screening compounds that specifically bind with a complex comprising a bHLH-PAS/JHR polypeptide and a heteromultimeric partner of said polypeptide, comprising:

(a) incubating a test compound in a solution that comprises an isolated bHLH-PAS/JHR polypeptide, and an isolated heteromultimeric partner of said polypeptide, and

(b) detecting the binding of said test compound with said complex.

In such methods, the test compound may be detectably labeled. In addition, the binding of said test compound with said polypeptide may be detected in step (b) using a scintillation proximity assay. Furthermore, in such a method, the detectably labeled test compound may comprise a detectable label selected from the group consisting of radiolabel, fluorescent label, chemiluminescent label, and bioluminescent label.

Such binding methods also may further comprise the step of incubating the bHLH-PAS/JHR polypeptide with a detectably labeled ligand, wherein said detectably

labeled ligand is added to said solution containing said receptor at a time selected from the group consisting of (i) prior to step (a), (ii) after step (a) and before step (b), and (iii) concomitantly with the addition of said test compound.

The detectably labeled ligand is these binding methods may be juvenile hormone or a juvenile hormone analog, and the detectable label may be selected from the group consisting of radiolabel, fluorescent label, chemiluminescent label, and bioluminescent label. Additionally, these methods may be carried out with [^{3}H]10*R*-juvenile hormone III or [^{3}H]methoprene.

These binding methods may also further comprise the step of incubating said bHLH-PAS/JHR polypeptide with a detectably labeled photoaffinity analog of juvenile hormone after step (a) and before step (b).

The binding methods of the invention may be carried out with a bHLH-PAS/JHR polypeptide selected from the group consisting of:

(a) a conservative amino acid variant of SEQ ID NO:4,

(b) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:4,

(c) a polypeptide having an amino acid sequence of SEQ ID NO:4,

(d) a conservative amino acid variant of SEQ ID NO:5,

(e) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:5,

(f) a polypeptide having an amino acid sequence of SEQ ID NO:5, and

(g) a *Met*-JHR alternatively-spliced isoform.

The invention further comprises a nucleic acid probe for detecting RFLPs in an insect population, wherein said RFLPs discriminate between JH-sensitive and JH-resistant individuals, said probe comprising a genetic locus in a

gene encoding a bHLH-PAS/JHR polypeptide that is associated with JH analog sensitivity and resistance traits.

The invention also encompasses a method for detecting JH-resistant individuals in an insect population, said method comprising:

(a) obtaining a representative biological sample of said population; and

(b) detecting a nucleic acid sequence in said sample that corresponds to a predetermined sequence within a gene encoding a bHLH-PAS/JHR polypeptide that is altered in JH analog-resistant individuals.

The detection step (b) may method comprise:

(i) amplifying a nucleic acid sequence from said sample, wherein said sequence corresponds to a predetermined sequence within a gene sequence encoding a bHLH-PAS/JHR polypeptide and wherein said sequence comprises at least one RFLP characteristic of JH analog resistance;

(ii) incubating said amplified nucleic acid with at least one predetermined restriction endonuclease, to form fragments;

(iii) size-separating said fragments to form a detectable pattern; and

(iv) comparing said pattern with a predetermined pattern obtained from JH analog-resistant individuals to detect the appearance of one or more RFLP characteristic of JH analog resistance.

The invention provides an *in vivo* method for screening compounds that specifically bind with a bHLH-PAS/JHR, comprising:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising a bHLH-PAS/JHR polypeptide and a second polypeptide comprising a DNA binding domain, and (2) a reporter gene under the control

of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said bHLH-PAS/JHR by monitoring expression of the reporter gene.

The invention further provides an *in vivo* method for screening compounds that specifically bind with a bHLH-PAS/JHR, comprising the steps of:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising a bHLH-PAS/JHR polypeptide and a second polypeptide comprising a DNA binding domain; (2) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide; and (3) DNA encoding a polypeptide that is a heterodimeric partner of said bHLH-PAS/JHR;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said bHLH-PAS/JHR by monitoring expression of the reporter gene.

The invention also provides an *in vivo* method for screening compounds that specifically bind to a multimeric complex comprising a bHLH-PAS/JHR polypeptide and the heteromultimeric partner of said polypeptide, comprising the steps of:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising bHLH-PAS/JHR polypeptide and the DNA binding domain of a second polypeptide, (2) DNA encoding a heteromultimeric partner of said bHLH-PAS/JHR polypeptide and the activation domain of said second polypeptide, and (3) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said complex by monitoring expression of the reporter gene.

The invention additionally provides an *in vivo* method for screening compounds that specifically bind to a multimeric complex comprising a bHLH-PAS/JHR polypeptide and the heteromultimeric partner of said polypeptide, comprising the steps of:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising bHLH-PAS/JHR polypeptide and the activation domain of a second polypeptide, (2) DNA encoding a heteromultimeric partner of said bHLH-PAS/JHR polypeptide and the DNA binding domain of said second polypeptide, and (3) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said complex by monitoring expression of the reporter gene.

The invention provides an *in vivo* method for screening compounds that specifically bind with a bHLH-PAS/JHR polypeptide, comprising:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising a bHLH-PAS/JHR polypeptide and the DNA binding region of a second polypeptide, (2) DNA encoding a bHLH-PAS/JHR polypeptide and the activation domain of said second polypeptide, and (3) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound with said bHLH-PAS/JHR polypeptide by monitoring expression of the reporter gene.

Any of the *in vivo* methods provided for by the invention may be employed using a host cell selected from the group of an insect cell, a yeast cell, and a mammalian cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the genomic region surrounding the P-element insertion sites in two mutant alleles, Met^{A3} and Met^{KI7}, the sequence which encodes bHLH-PAS/JHR, and the transcripts observed for this region. The figure also shows DNA fragments used in transformation studies to rescue the *Met* phenotype, demonstrating that fragment St-H carries a functional copy of the *Met* gene. P-element insertional sites in the Met^{A3} and Met^{KI7} alleles are shown at the arrows. The locations of the transcripts as deduced from cDNA sequencing and RT-PCR analysis are noted below the map. The genomic transformation fragments are indicated. Those that did not rescue the resistance phenotype are noted (-) and the fragment that produced methoprene susceptibility in transformant flies is noted (+). D = *Hind* III; S = *Sal* I; K = *Kpn* I; St = *Stu* I; B = *Bam* HI; X = *Xho* I; H = *Hpa* I.

Figure 2 (SEQ ID NO:1) shows the nucleotide sequence of the 6.234 Kb St-H segment shown in Figure 1. Base number 1514, A, is underlined and designates the first base of the *Met*-JHR open reading frame. Base number 3732, G, is underlined and designates the last base of the *Met*-JHR open reading frame. Base number 1292, C, is underlined and is the first base in the genomic DNA sequence in Figure 3. Base number 4301, T, is underlined and designates the last base in the genomic DNA sequence in Figure 3. The intron is shown in lower case letters.

Figures 3A and 3B provide the nucleotide sequences for the *Met*-JHR genomic DNA ("MetGen"; SEQ ID NO:2) and cDNA (SEQ ID NO:3). Boxed residues are those in the cDNA that differ from the genomic DNA. Nucleotide 875 in the genomic DNA is designated as "R," indicating that a G or a C may be present in this position. If it is G, the corresponding amino acid is Gly (G) and if it is C, the corresponding amino acid is Arg (R). Nucleotide 526 in the cDNA is designated as "M," indicating that an A or a C may be present in this position. If the nucleotide is A, the corresponding amino acid is Thr (T), and if it is C, the corresponding amino acid is Pro (P).

Figure 4 provides the amino acid sequences deduced from the *Met*-JHR genomic DNA (SEQ ID NO:4) and cDNA (SEQ ID NO:5). "X" at amino acid 103 deduced from cDNA means that this residue may be Gly or Arg. "X" at amino acid 218 deduced from the genomic DNA means that Thr or Pro.

Figure 5 shows a comparison of a portion of the *Met*-JHR gene from *D.melanogaster* (sequence A) (SEQ ID NO:6) and a nucleotide sequence from the *Met* gene from *D.erecta* (sequence B) (SEQ ID NO:7). Dash symbols (-) indicate spaces in the printed sequence that were added to show alignment of the A and B sequences.

Figure 6 provides a comparison of the amino acid sequence of the *Met*-JHR cDNA, *Drosophila* aromatic hydrocarbon receptor nuclear translocator protein (DARNT), human ARNT (HARNT) [Zelzer *et al. Genes & Dev.* 11:2079 (1997)], brain and muscle ARNT-like protein a (BmAl1) [Ikeda *et al. Biochem. Biophys. Res. Comm.* 233:258 (1997)] and human aromatic hydrocarbon receptor (AhR-human). (SEQ ID NOS: 8-12). The motifs of bHLH-PAS proteins [Rowlands *et al. Crit. Rev. Toxicol.* 27:109 (1997)] that are functionally characterized for ARNT are included at the top of the alignments. The residues of *Met*-JHR are boxed, and the residues in the other four proteins that match *Met*-JHR also are boxed. When an

amino acid residue from all five proteins match, they form a consensus sequence which is included at the top of the alignments. The skilled artisan will recognize that additional matches can be generated by moving amino acids one or two positions. The position of the "LXXLL" motif in the *Met*-JHR gene is also shown, above the appropriate sequence, LMQLL (amino acids 129-133).

Figure 7 shows *in vitro* transcription and translation of the *Met*-JHR cDNA to produce a protein band that migrates at about 78,000 daltons. The reaction uses T3 or T7 polymerase, in the presence (+) or absence (-) of microsomal membranes. The *Met*-JHR gene is transcribed only by the T7 polymerase (lanes 2 and 4) and it does not appear to be post-translationally modified (proteolytically processed or glycosylated) by the microsomal membranes (lane 2 *vs.* lane 4).

Figure 8 shows the Northern blot of total RNA isolated from larvae homozygous for *v* or any of various other *Met* alleles, probed with a 331 bp riboprobe. *Met*, Met^{2}, Met^{3} are EMS-induced alleles; Met^{A3} and Met^{KI7} are P-element alleles, and the remaining alleles were X-ray induced from methoprene-susceptible *vermillion* (*v*) flies.

Figure 9 shows a developmental Northern of total RNA isolated from the methoprene-susceptible Oregon-RC strain at various times in development, probed with the 331 bp riboprobe. The indicated times for larvae are +/- 8 hours.

DETAILED DESCRIPTION

1. Overview

Attempts have been made to rationally design a potent JH analog, but the structure-activity relationship was found to be extremely complex, and varied from species to species. Retnakaran *et al.*, "Insect Growth Regulators," in COMPREHENSIVE INSECT PHYSIOLOGY AND PHARMACOLOGY,

Volume 12, Kerkut *et al.* (eds.) pages 530-601 (Pergamon Press 1985). In retrospect, the chemical structures of many JH analogs were found to be totally unrelated to the structures of endogenous juvenile hormone. See, for example, Sláma, "Pharmacology of Insect Juvenile Hormones," in COMPREHENSIVE INSECT PHYSIOLOGY AND PHARMACOLOGY, Volume 11, Kerkut *et al.* (eds.) pages 357-394 (Pergamon Press 1985). Accordingly, novel insecticides that interfere with JH action were primarily discovered by an almost random testing of thousands of chemical compounds for efficacy against insects in bioassays.

JH analogs will maintain insects in an immature state. Thus, JH analog insecticides will be most useful in combatting insects that do not have a destructive immature stage (*e.g.* a larval stage), that will damage crops. On the other hand, some insects have highly destructive larval stages, such as caterpillars. Thus, it is not desirable to maintain such insects in the immature state. JH antagonists will be useful as insecticides. A JH antagonist will be lethal during the larval phase, "tricking" into entering pupation which the larva is not equipped to handle.

As described herein, a novel bHLH-PAS protein has been isolated from *Drosophila*, and is called *Met*-JHR. It is expected that the *Met*-JHR gene encodes a JH receptor. Various features of the *Met*-JHR gene are consistent with all the features predicted by biochemical analysis of the JHR protein [Shemshedini *et al. J. Biol. Chem.* 265(4):1913 (1990)] and the *Met*-JHR gene product. The longest single open reading frame in the genomic *Met*-JHR sequence encodes a protein of 78,720 daltons and has the structure of a bHLH-PAS nuclear transcriptional protein.

The *Met*-JHR gene is used to express polypeptide useful for *in vitro* and *in vivo* screening of insecticides. The gene also is useful for isolating

related bHLH-PAS/JHR genes from a variety of insects, which in turn, can be used for species-specific screening assays. Having such genes permits a more detailed analysis of the mechanisms of ligand binding, and hence rational design of insecticides. Such genes also will permit monitoring insects for JHIII and JHIII analog resistance.

2. Definitions

In the description that follows, a number of terms are utilized extensively. Definitions are herein provided to facilitate understanding of the invention.

Structural gene. A DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide (protein).

Promoter. A DNA sequence which directs the transcription of a structural gene to produce mRNA. Typically, a promoter is located in the 5' region of a gene, proximal to the start codon of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Enhancer. A genetic element related to transcription. An enhancer can increase the efficiency with which a particular gene is transcribed into mRNA irrespective of the distance or orientation of the enhancer relative to the start site of transcription. The enhancer effect is mediated through sequence-specific DNA binding proteins. An enhancer is also referred to as a "response element."

Complementary DNA (cDNA). Complementary DNA is a single-stranded DNA molecule that can be formed from an mRNA template by the enzyme reverse transcriptase.

Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule derived from a single mRNA molecule.

Genomic DNA. Chromosomal DNA, including introns. An intron is an intervening sequence. It is a non-coding sequence of DNA within a gene that is transcribed into *hn*RNA but is then removed by RNA splicing in the nucleus, leaving a mature mRNA which is then translated in the cytoplasm. The regions at the ends of an intron are self-complementary, allowing a hairpin structure to form naturally in the *hn*RNA.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Cloning vector. A DNA molecule, such as a plasmid, cosmid, phagemid, or bacteriophage or other virally-derived entity, which has the capability of replicating autonomously in a host cell and which is used to transform cells for gene manipulation. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences may be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene which is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

***Drosophila* mutants.** The mutant gene that is responsible for methoprene resistance is termed *Methoprene-tolerant*, symbolized as *Met*. Various mutant alleles are given superscripts; for example, Met^{43}. *Met* implies Met^{1}, the original mutant allele recovered, when

speaking of the mutant fly. The wild-type or normal gene is termed *Met*$^{+}$ and describes the genotype in flies that have a normally functioning bHLH-PAS/JHR protein.

Expression vector. A DNA molecule comprising a cloned structural gene encoding a foreign protein which provides the expression of the foreign protein in a recombinant host. Typically, the expression of the cloned gene is placed under the control of (*i.e.*, operably linked to) certain regulatory sequences such as promoter and enhancer sequences. Promoter sequences may be either constitutive or inducible.

Recombinant Host. A recombinant host may be any prokaryotic or eukaryotic cell which contains either a cloning vector or expression vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell. For examples of suitable hosts, see Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989) ["Sambrook"].

As used herein, a **"substantially pure protein"** means that the desired purified protein is essentially free from contaminating cellular components, as evidenced by a single band following polyacrylamide-sodium dodecyl sulfate gel electrophoresis (SDS-PAGE).

The term "substantially pure" is further meant to describe a molecule which is homogeneous by one or more purity or homogeneity characteristics used by those of skill in the art. For example, a substantially pure bHLH-PAS/JHR will show constant and reproducible characteristics within standard experimental deviations for parameters such as the following: molecular weight, chromatographic migration, amino acid composition, amino acid sequence, blocked or unblocked N-terminus, HPLC elution profile, biological activity, and other such

parameters. The term, however, is not meant to exclude artificial or synthetic mixtures of the molecule with other compounds. In addition, the term is not meant to exclude bHLH-PAS/JHR fusion proteins isolated from a recombinant host.

Juvenile Hormone. The members of the JH family are: JH I ([2*R*-[2α(2*E*,6*E*),3α]]-7-ethyl-9-(3-ethyl-3-methyloxiranyl)-3-methyl-2,6-nonadienoic acid methyl ester; methyl (2*E*,6*E*,10*R*,11*S*)-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate; C-18 JH), JH II ([2*R*-[2α(2*E*,6*E*),3α]]-9-(3-ethyl-3-methyloxiranyl)-3,7-dimethyl-2,6-nonadienoic acid methyl ester; methyl (2*E*,6*E*,10*R*,11*S*)-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate; C-17 JH), and JH III ([*R*-(*E*,*E*)]-9-(3,3-dimethyloxiranyl)-3,7-dimethyl-2,6-nonadienoic acid methyl ester; methyl (2*E*,6*E*,10*R*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate; C-16 JH).

Juvenile Hormone Analog. A JH analog is a compound that is an agonist -- it mimics JH and usually has insecticidal properties resulting from this activity. Examples of JH analogs include: methoprene ([*E*,*E*]-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid 1-methylethyl ester) and pyriproxyfen (2-[1-methyl-2-(4-phenoxyphenoxy)ethoxylpyridine).

Juvenile Hormone Antagonist. A compound that will block the activity of JH and that can have insecticidal properties resulting from this activity. An antagonist prevents JH agonists from eliciting their effects.

3. Isolation of DNA Sequences that Encode the *Met* Juvenile Hormone Receptor

To study the role of juvenile hormone, a genetic approach was used to identify an insensitive JH mutant. These studies took advantage of the high toxicity of methoprene, a JH analog insecticide to *Drosophila*.

Wilson and Fabian, *Dev. Biol. 118*:190 (1986); Riddiford and Ashburner, *Gen. Comp. Endocrinol. 82*:172 (1991). Reasoning that the phenotype "resistance to methoprene" would produce a mutant that also would be resistant to JH, and that the primary lesion potentially could be in a JHR, progeny of *Drosophila* males that had been mutagenized by ethyl methanesulfonate (a chemical mutagen) or X-rays were screened on a dose of methoprene that is toxic to susceptible flies. Wilson and Fabian, "Selection of methoprene-resistant mutants of *Drosophila melanogaster*," in Law (ed.), MOLECULAR ENDOCRINOLOGY. UCLA SYMPOSIA ON MOLECULAR AND CELLULAR BIOLOGY, NEW SERIES, Volume 49, pages 179-188 (1987). A total of eight dominant *Drosophila* lines with high resistance to methoprene were recovered, all of which proved to be alleles at a locus designated as *Methoprene-tolerant* (*Met*). Wilson and Fabian, (1986, 1987).

The mutant *Met* phenotype has been genetically characterized as follows: (1) *Met* results in as much as 100-fold resistance to both the toxic and morphogenetic effects of methoprene; (2) *Met* maps by recombination to 35.4 on the X-chromosome and by deficiency mapping to polytene chromosome bands 10C5-D2; (3) loss of wild-type *Met* gene function is expressed as a semidominant mutation; resistance is present in heterozygotes at a level intermediate between that in homozygotes and in wild-type; and (4) the *Met* gene mutation results in resistance to topical application of both the natural hormones, JH III and JH bisepoxide, as well as to two additional JH analogs, fenoxycarb and pyriproxyfen. Mutant *Met* flies are not resistant to other classes of insecticides that have different modes of action. The *Met* phenotype was also found to be expressed autonomously in genetic mosaics. This observation ruled out a circulating factor as the basis of *Met* resistance. Wilson and Fabian (1986).

The biochemistry of *Met* resistance has been studied extensively. Biochemical analysis of *Met* resistance has eliminated four, and identified one, possible mechanisms for resistance. Enhanced secretion or metabolism, tissue sequestration, and reduced cuticular penetration of JH were ruled out by direct experimentation. Shemshedini and Wilson, *Proc. Nat'l Acad. Sci. USA* 87:2072 (1990). However, when binding of JH to a target tissue was examined, *Met* flies were found to possess a JH cytosolic binding protein that has an apparent 10-fold lower binding affinity for JH III than that from Met^{+} flies. Similar results with lowered affinity JH binding proteins were obtained upon examination of two additional *Met* alleles. Shemshedini and Wilson, *Proc. Nat'l Acad. Sci. USA* 87:2072 (1990).

An initial experiment was designed to clone the *Met* gene by transposon tagging with P-element transposable genetic elements. Bingham *et al.*, *Cell* 25:693 (1981). This method required a P-element insertion either in or near the Met^{+} (wild type) gene. A screen was devised to recover P-element insertional *Met* alleles following P-element-mediated mutagenesis, and four alleles were recovered. Two of these, designated Met^{A3} and Met^{K17}, were shown to be P-element insertions by both genetic reversion experiments and *in situ* hybridization of a P-element DNA probe to the expected cytogenetic region of *Met* at 10C expected for the Met^{+} gene. Wilson *et al.*, "Molecular analysis of *Methoprene-tolerant*, a gene in *Drosophila* involved in resistance to JH analog insect growth regulators," in MOLECULAR MECHANISMS OF INSECTICIDE RESISTANCE: DIVERSITY AMONG INSECTS. AMERICAN CHEMICAL SOCIETY SYMPOSIUM SERIES, Volume 505, Mullin *et al.* (eds), pages 99-112 (1992); Wilson, *J. Econ. Entomol.* 86:645 (1993). Each of the Met^{A3} and Met^{K17} alleles conferred resistance to both the toxic and morphogenetic effects JH and methoprene, and susceptible

revertants could be recovered by genetic means. Wilson, *et al. Mol. Mech. of Insecticide Resistance* (Am. Chem. Soc. Symp.) 505:99 (1993); Wilson, T.G. *J. Econ. Entomol.* 86:645 (1993).

In a follow-up study, genomic libraries were constructed from these alleles to isolate a large region that likely contains the *Met* gene. Turner and Wilson, *Arch. Insect Biochem. Physiol. 30*:133 (1995). cDNA molecules from a late-larval cDNA library were also obtained from this region. One transcriptional unit was found to be located very close to the P-element insertion site.

As described herein, cDNA molecules encoding the *Met* JHR gene were isolated from a *Drosophila* ovary cDNA library. The genomic DNA for the *Met* gene was found in a 6.234 Kb St-H fragment, which is shown in Figure 2 (SEQ ID NO:1).

By comparing the genomic DNA with a cDNA of 3.282 Kb, it was deduced that the genomic DNA sequence includes a 2.22 Kb open reading frame that is divided by one intron of 69 nucleotides (bases 1520 to 1588). SEQ ID NO:2. A 3.282 kB sequence that contains a *Met* JHR gene cDNA is shown in Figure 3 (SEQ ID NO:3). The cDNA was used to transcribe and translate a protein that approximates the predicted size of the *Met* cDNA open reading frame, as shown in Figure 7.

The 6.234 Kb segment comprises the intron (lower case letters; bases 2809-2878) and the *Met*-JHR open reading frame (base no. 1514 to base no. 3732). Also shown in Figure 1 are the first (no. 1292) and last (no. 4301) bases of the genomic *Met*-JHR sequence in Figure 3. The invention thus comprises isolated polynucleotides comprising a DNA sequence from base no. 1 though base no. 1291 of SEQ ID NO:1, or a DNA sequence from base no. 1 though base no. 1513 of SEQ ID NO:1, or a DNA sequence from base no. 3733 through base no. 6235 of SEQ ID NO:1,

or a DNA sequence from base no. 4302 through base no. 6235 of SEQ ID NO:1.

Polynucleotides encoding the *Met*-JHR protein are obtained by screening cDNA or genomic libraries with polynucleotide probes having nucleotide sequences based upon SEQ ID NO:1 or SEQ ID NO:2. *Drosophila melanogaster* cDNA and genomic libraries are constructed according to standard methods. Optionally, libraries are obtained from commercial sources, such as the American Type Culture Collection (*e.g.*, ATCC 37332 is a *D. melanogaster* genomic library).

Alternatively, the *Met* JHR gene is obtained by synthesizing polynucleotides using mutually priming long oligonucleotides. See, for example, Ausubel *et al.*, (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, pages 8.2.8 to 8.2.13 (1990) ["Ausubel"]. Also, see Wosnick *et al.*, *Gene 60*:115 (1987); and Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 8-8 to 8-9 (John Wiley & Sons, Inc. 1995). Established techniques using the polymerase chain reaction provide the ability to synthesize polynucleotides at least 2 kilobases in length. Adang *et al.*, *Plant Molec. Biol. 21*:1131 (1993); Bambot *et al.*, *PCR Methods and Applications 2*:266 (1993); Dillon *et al.*, "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in METHODS IN MOLECULAR BIOLOGY, Vol. 15: PCR PROTOCOLS: CURRENT METHODS AND APPLICATIONS, White (ed.), pages 263-268, (Humana Press, Inc. 1993); Holowachuk *et al.*, *PCR Methods Appl. 4*:299 (1995).

The invention further comprise nucleotide sequences that hybridize with a *Met*-JHR polynucleotide of the invention under stringent conditions. Suitable hybridization conditions are discussed below.

"Hybridization" is used here to denote the pairing of complementary nucleotide sequences to produce a DNA-DNA hybrid or a DNA-RNA hybrid. Complementary base

sequences are those sequences that are related by the base-pairing rules. In DNA, A pairs with T and C pairs with G. In RNA U pairs with A and C pairs with G.

Typically, nucleotide sequences to be compared by means of hybridization are analyzed using dot blotting, slot blotting, Northern or Southern blotting. Southern blotting is used to determine the complementarity of DNA sequences. Northern blotting determines complementarity of DNA and RNA sequences. Dot and Slot blotting can be used to analyze DNA/DNA or DNA/RNA complementarity. These techniques are well known by those of skill in the art. Typical procedures are described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.*, eds.) (John Wiley & Sons, Inc. 1995) at pages 2.9.1 through 2.9.20.

A probe is a biochemical labeled with a radioactive isotope or tagged in other ways for ease in identification. A probe is used to identify a gene, a gene product or a protein. Thus a polynucleotide probe can be used to identify complementary nucleotide sequences. An mRNA probe will hybridize with its corresponding DNA gene. An antisense "riboprobe" also will hybridize to is corresponding DNA gene.

Typically, the following general procedure is used to determine hybridization under stringent conditions. A *Met*-JHR polynucleotide according to the invention is immobilized on a membrane. A sample polynucleotide will be labeled and used as a "probe." Using procedures well known to those skilled in the art for blotting described above, the ability of the probe to hybridize with a nucleotide sequence according to the invention can be analyzed. Conversely, the sample polynucleotide is immobilized and a *Met*-JHR polynucleotide is used as a probe.

One of skill in the art will recognize that various factors can influence the amount and detectability of the

probe bound to the immobilized DNA. The specific activity of the probe must be sufficiently high to permit detection. Typically, a specific activity of at least 10^8 dpm/ug is necessary to avoid weak or undetectable hybridization signals when using a radioactive hybridization probe. A probe with a specific activity of 10^8 to 10^9 dpm/ug can detect approximately 0.5 pg of DNA. It is well known in the art that sufficient DNA must be immobilized on the membrane to permit detection. It is desirable to have excess immobilized DNA and spotting 10ug of DNA is generally an acceptable amount that will permit optimum detection in most circumstances. Adding an inert polymer such as 10% (w/v) dextran sulfate (mol. wt. 500,000) or PEG 6000 to the hybridization solution can also increase the sensitivity of the hybridization. Adding these polymers has been known to increase the hybridization signal. See Ausubel, *supra*, at p 2.10.10.

To achieve meaningful results from hybridization between a first nucleotide sequence immobilized on a membrane and a second nucleotide sequence to be used as a hybridization probe, (1) sufficient probe must bind to the immobilized DNA to produce a detectable signal (sensitivity) and (2) following the washing procedure, the probe must be attached only to those immobilized sequences with the desired degree of complementarity to the probe sequence (specificity).

"Stringency," as used in this specification, means the condition with regard to temperature, ionic strength and the presence of certain organic solvents, under which nucleic acid hybridizations are carried out. The higher the stringency used, the higher degree of complementarity between the probe and the immobilized DNA.

"Stringent conditions" designates those conditions under which only polynucleotides that have a high frequency of complementary base sequences will hybridize with each other.

Exemplary stringent conditions are (1) 0.75 M dibasic sodium phosphate/0.5 M monobasic sodium phosphate/1 mM disodium EDTA/1% sarkosyl at about 42°C for at least about 30 minutes, (2) 6.0M urea/0.4% sodium laurel sulfate/0.1% SSX at about 42° C for at least about 30 minutes, (3) 0.1X SSC/0.1% SDS at about 68°C for at least about 20 minutes, (4) 1X SSC/0.1% SDS at about 55°C for about one hour, (5) 1X SSC/0.1% SDS at about 62°C for about one hour, (6) 1X SSC/0.1% SDS at about 68°C for about one hour, (7) 0.2X SSC/0.1% SDS at about 55°C for about one hour, (8) 0.2X SSC/0.1% SDS at about 62°C for about one hour, and (9) 0.2X SSC/0.1% SDS at about 68°C for about one hour. *See, e.g.* CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.*, eds.) (John Wiley & Sons, Inc. 1995), pages 2.10.1-2.10.16 of which are hereby incorporated by reference and Sambrook, *et al.*, MOLECULAR CLONING. A LABORATORY MANUAL (Cold Spring Harbor Press, 1989) at §§1.101-1.104.

While stringent washes are typically carried out at temperatures from about 42°C to about 68°C, one of skill in the art will appreciate that other temperatures may be suitable for stringent conditions. Maximum hybridization typically occurs at about 20 to about 25°C below the T_m for DNA-DNA hybrids. It is well known in the art that T_m is the melting temperature, or temperature at which two nucleotide sequences dissociate. Methods for estimating T_m are well known in the art. *See, e.g.* Ausubel, *supra*, at page 2.10.8. Maximum hybridization typically occurs at about 10 to about 15°C below the T_m for DNA-RNA hybrids.

Naturally occurring variants of the *Met*-JHR gene and protein are included in the present invention. For example, variants of the *Met*-JHR gene are the result of naturally-occurring polymorphisms. For example, the genomic and cDNA for the *Met*-JHR differ by one amino acid residue due to polymorphism. Amino acid No. 274 is R in

the genomic DNA and the corresponding amino acid in the cDNA is W. Other nucleotide differences between the genomic and cDNA are boxed in Figure 3.

Sequence ambiguities also give rise to variants of the *Met*-JHR. In this regard, amino acid no. 103 deduced from the *Met*-JHR cDNA can be T or P. In the corresponding genomic DNA, amino acid no. 218 can be G or R. In addition, variants of the *Met* gene result from intron diversity. As used herein, "a *Met*-JHR alternatively-spliced isoform" is used to designate an isoform of the *Met*-JHR gene that results from alternate splicing due to the presence of an intron in this gene. In one isoform, there is no splicing and there is read through to the first stop codon, to produce a 1320 nucleotide-long sequence. This encodes a 439 amino acid protein (439 amino acids + TAA stop codon).

The skilled artisan will recognize that potential introns can be identified using various computer programs. Using one system, potential donor sites were recognized at nucleotides 549, 1517, 1586, and 2147 of the *Met*-JHR genomic DNA (3011 nucleotides). Acceptor sites were recognized at positions 260, 278, 651, 875, 1071, and 1192. Using a second system, donor sites in the *Met*-JHR open reading frame were identified at positions 320-334, 1288-1302, 1357-1371, 1431-1445, 1546-1560, and 1918-1932. Acceptor sites were identified at 274-314, 436-476, 494-534, 829-869, 950-990, 1676-1716, 1716-1756, 2009-2049 and 2076-2116. Using data such as this, potential alternatively spliced isoforms of the *Met*-JHR gene can be identified using routine optimization.

Additionally, variants of the *Met*-JHR can be produced that contain conservative amino acid changes, compared with the parent receptor molecule. That is, variants can obtained that contain one or more amino acid substitutions of SEQ ID NO:4 or SEQ ID NO:5, in which an

alkyl amino acid is substituted for an alkyl amino acid in the *Met* JHR amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in the *Met* JHR amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in the *Met* JHR amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in the *Met* JHR amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in the *Met* JHR amino acid sequence, a basic amino acid is substituted for a basic amino acid in the *Met* JHR amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in the *Met* JHR amino acid sequence. Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) cysteine and methionine, (4) serine and threonine, (5) aspartate and glutamate, (6) glutamine and asparagine, and (7) lysine, arginine and histidine. Of course other amino acid substitutions can be undertaken.

Conservative amino acid changes in the *Met* JHR can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO:2 or SEQ ID NO:3. Such "conservative amino acid" variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like. Ausubel *et al.*, *supra*, at pages 8.0.3-8.5.9; Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 8-10 to 8-22 (John Wiley & Sons, Inc. 1995). Also see generally, McPherson (ed.), DIRECTED MUTAGENESIS: A PRACTICAL APPROACH, IRL Press (1991). The ability of such variants

to bind JH or an analog can be determined using any of the standard binding assays described herein.

In addition, routine deletion analyses can be performed to obtain "functional fragments" of the *Met* JHR. As an illustration, polynucleotides having the nucleotide sequence of SEQ ID NO:2 or 3 can be digested with *Bal*31 nuclease to obtain a series of nested deletions. The fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptide are isolated and tested for the ability to bind JH or an analog using a standard assay. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired fragment. Alternatively, particular fragments of a bHLH-PAS/JHR gene can be synthesized using the polymerase chain reaction. Standard techniques for functional analysis of proteins are described by, for example, Treuter *et al.*, *Molec. Gen. Genet.* *240*:113 (1993); Content *et al.*, "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in BIOLOGICAL INTERFERON SYSTEMS, PROCEEDINGS OF ISIR-TNO MEETING ON INTERFERON SYSTEMS, Cantell (ed.), pages 65-72 (Nijhoff 1987); Herschman, "The EGF Receptor," in CONTROL OF ANIMAL CELL PROLIFERATION, Vol. 1, Boynton *et al.*, (eds.) pages 169-199 (Academic Press 1985); Coumailleau *et al.*, *J. Biol. Chem.* *270*:29270 (1995); Fukunaga *et al.*, *J. Biol. Chem.* *270*:25291 (1995); Yamaguchi *et al.*, *Biochem. Pharmacol.* *50*:1295 (1995); and Meisel *et al.*, *Plant Molec. Biol.* *30*:1 (1996).

The skilled artisan will recognize that it is a matter of routine optimization to perform deletion analysis of the *Met*-JHR gene to ascertain the functions that are associated with certain domains, and to obtain corresponding functional fragments. One way to identify

such fragments is to create chimeric proteins by swapping functional domains between bHLH-PAS/JHR and other members of the bHLH-PAS family. As explained in Example 2, the *Met*-JHR gene contains structures (sequences) that are associated with conserved functions in other proteins. For example, the bHLH region is involved in DNA binding and heterodimerization. The P/S/T region is involved in transactivation. Other regions are described in Example 2. Thus, the invention encompasses fragments of the *Met*-JHR gene that encode proteins that have one or more of these functions.

The present invention also contemplates functional fragments of Met gene that have conservative amino acid changes.

4. Expression of the Cloned *Met* Juvenile Hormone Receptor

To express the polypeptide encoded by the *Met* JHR gene, the DNA sequence must be operably linked to regulatory sequences controlling transcriptional expression in an expression vector and then, introduced into either a prokaryotic or eukaryotic host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Suitable promoters for expression in a prokaryotic host can be repressible, constitutive, or inducible. Suitable promoters are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lac*λ*pr*, *phoA*, and *lacZ* promoters of *E. coli*, the α-amylase and the σ^{28}-specific promoters of *B. subtilis*, the promoters of the bacteriophages of

Bacillus, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol.* 1:277 (1987); Watson *et al.*, MOLECULAR BIOLOGY OF THE GENE, 4th Ed., Benjamin Cummins (1987); Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*.

A preferred prokaryotic host is *E. coli*. Suitable strains of *E. coli* include DH1, DH4α, DH5, DH5α, DH5αF', DH5αMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (Ed.), MOLECULAR BIOLOGY LABFAX, Academic Press (1991)). An alternative preferred host is *Bacillus subtilus*, including such strains as BR151, YB886, MI119, MI120, and B170. See, for example, Hardy, "Bacillus Cloning Methods," in DNA CLONING: A PRACTICAL APPROACH, Glover (Ed.), IRL Press (1985).

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art. See, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 15-58 (Oxford University Press 1995). Also see, Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, pages 137-185 (Wiley-Liss, Inc. 1995); and Georgiou, "Expression of Proteins in Bacteria," in PROTEIN ENGINEERING: PRINCIPLES AND PRACTICE, Cleland *et al.* (eds.), pages 101-127 (John Wiley & Sons, Inc. 1996).

Expression vectors that are suitable for production of bHLH-PAS/JHR protein in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance

marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence.

A bHLH-PAS/JHR protein of the present invention can be expressed in insect, mammalian, and yeast cells. Preferably, receptor protein is produced in insect cells using a baculovirus system. Recombinant proteins expressed by baculoviruses in insect cells undergo correct posttranslational modification, including glycosylation, phosphorylation, palmitylation, myristylation, signal peptide cleavage, and intracellular transport. Suitable expression vectors are based upon the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as *Drosophila* heat shock protein (hsp) 70 promoter, *Autographa californica* nuclear polyhedrosis virus immediate-early gene promoter (*ie-1*) and the delayed early *39K* promoter, baculovirus p10 promoter, baculovirus polyhedrin promoter, and the *Drosophila* metallothionein promoter. Suitable insect host cells include cell lines derived from IPLB-*Sf*-21, a *Spodoptera frugiperda* pupal ovarian cell line, such as *Sf*9 (ATCC CRL 1711), *Sf*21AE, and *Sf*21 (Invitrogen Corporation; San Diego, CA), as well as *Tricoplusia ni* 5B14 cells, and *Drosophila* Schneider-2 cells. Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey *et al.*, "Manipulation of Baculovirus Vectors," in METHODS IN MOLECULAR BIOLOGY, Volume 7: GENE TRANSFER AND EXPRESSION PROTOCOLS, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel *et al.*, "The baculovirus expression system," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel *et al.* (eds.), SHORT

PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 16-37 to 16-57 (John Wiley & Sons, Inc. 1995), by Richardson (ed.), BACULOVIRUS EXPRESSION PROTOCOLS (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in PROTEIN ENGINEERING: PRINCIPLES AND PRACTICE, Cleland *et al.* (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Suitable yeast expression vectors include, but are not limited to, YEp and YIp vectors. Hill *et al. Yeast* 2:163 (1986). Any suitable recombinant cloning vectors may be used for introducing foreign DNA sequences into yeast. Such vectors may include one or more replication systems for cloning or expression, one or more markers for selection in the host (*e.g.*, prototrophy or antibiotic resistance) and one or more expression cassettes. Examples of yeast promoters include, but are not limited to, the metallothionein promoter (CUP1), triosephosphate dehydrogenase promoter (TDH3), 3-phosphoglycerate kinase promoter (PGK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter, galactokinase (GALI) promoter, galactoepimerase promoter and alcohol dehydrogenase (ADH) promoter.

Yeast host cells may be transformed using any suitable method, including, but not limited to, methods that employ calcium phosphate, lithium salts, electroporation, and spheroplast formation. Sherman *et al*, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory (1982). Suitable host cells include, but are not limited to, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

Examples of mammalian host cells include human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21; ATCC CRL 8544), canine kidney cells (MDCK; ATCC CCL 34), Chinese hamster ovary cells (CHO-K1; ATCC CCL61), rat pituitary cells (GH_1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma

cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as actin, collagen, myosin, and metallothionein genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse metallothionein I gene [Hamer *et al.*, *J. Molec. Appl. Genet. 1*:273 (1982)], the TK promoter of Herpes virus [McKnight, *Cell 31*:355 (1982)], the SV40 early promoter [Benoist *et al.*, *Nature 290*:304 (1981)], the Rous sarcoma virus promoter [Gorman *et al.*, *Proc. Nat'l Acad. Sci. USA 79*:6777 (1982), the cytomegalovirus promoter [Foecking *et al.*, *Gene 45*:101 (1980)], and the mouse mammary tumor virus promoter. See, generally, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in PROTEIN ENGINEERING: PRINCIPLES AND PRACTICE, Cleland *et al.* (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control fusion gene expression if the prokaryotic promoter is regulated by a eukaryotic promoter. Zhou *et al.*, *Mol. Cell. Biol. 10*:4529 (1990); Kaufman *et al.*, *Nucl. Acids Res. 19*:4485 (1991).

An expression vector can be introduced into host cells using a variety of techniques including calcium phosphate transfection, liposome-mediated transfection,

electroporation, and the like. Preferably, transfected cells are selected and propagated wherein the expression vector is stably integrated in the host cell genome to produce stable transformants. Techniques for introducing vectors into eukaryotic cells and techniques for selecting stable transformants using a dominant selectable marker are described, for example, by Ausubel and by Murray (ed.), GENE TRANSFER AND EXPRESSION PROTOCOLS (Humana Press 1991).

5. Isolation of the Cloned *Met* Juvenile Hormone Receptor and Production of Anti-Receptor Antibodies

(a) Isolation of Recombinant Receptor Protein

General methods for recovering protein produced by a bacterial system is provided by, for example, Grisshammer *et al.*, "Purification of over-produced proteins from *E. coli* cells," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 59-92 (Oxford University Press 1995). Established techniques for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), BACULOVIRUS EXPRESSION PROTOCOLS (The Humana Press, Inc. 1995).

bHLH-PAS/JHR proteins can be purified using standard methods that have been used to purify JH binding proteins, including gel filtration, ion exchange chromatography, isoelectric focusing, hydroxylapatite chromatography, and affinity chromatography. See, for example, Goodman *et al.*, "Development of Affinity Chromatography for Juvenile Hormone Binding Proteins," in JUVENILE HORMONE BIOCHEMISTRY, Pratt *et al.* (eds.), pages 365-374 (Elsevier/North-Holland Biomedical Press 1981); Goodman *et al.*, "Juvenile Hormone Cellular and Hemolymph Binding Proteins," in COMPREHENSIVE INSECT PHYSIOLOGY AND PHARMACOLOGY, Volume 7, Kerkut *et al.* (eds.) pages 491-510 (Pergamon Press 1985). Moreover, general affinity

chromatography techniques are provided by, for example, Dean *et al.*, AFFINITY CHROMATOGRAPHY: A PRACTICAL APPROACH (IRL Press 1985).

As an alternative, anti-*Met* JHR antibodies, obtained as described below, can be used to isolate large quantities of *Met* JHR by immunoaffinity purification.

(b) Preparation of Anti-Met Juvenile Hormone Receptor Antibodies and Fragments Thereof

Antibodies to *Met* JHR can be obtained using the product of an expression vector as an antigen. Polyclonal antibodies to such receptor protein can be prepared using methods well-known to those of skill in the art. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in IMMUNOCHEMICAL PROTOCOLS (Manson, ed.), pages 1-5 (Humana Press 1992). Also see, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 15-58 (Oxford University Press 1995).

Alternatively, an anti-*Met* JHR antibody can be derived from a rodent monoclonal antibody (MAb). Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art. *See*, for example, Kohler *et al.*, *Nature 256*:495 (1975), and Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"]. Also see, Picksley *et al.*, "Production of monoclonal antibodies against proteins expressed in *E. coli*," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 93-122 (Oxford University Press 1995)

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing

a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

MAbs can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3. Also, see Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992).

For particular uses, it may be desirable to prepare fragments of anti-*Met* JHR antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent Nos. 4,036,945 and 4,331,647 and references contained therein. Also, see Nisonoff *et al.*, *Arch Biochem. Biophys.* *89*:230 (1960); Porter, *Biochem. J.* 73:119 (1959), Edelman *et al.*, in

METHODS IN ENZYMOLOGY VOL. 1, page 422 (Academic Press 1967), and Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association can be noncovalent, as described in Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA* *69*:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. See, for example, Sandhu, *Crit. Rev. Biotech.* *12*:437 (1992).

Preferably, the Fv fragments comprise V_H and V_L chains which are connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow *et al.*, *Methods: A Companion to Methods in Enzymology* *2*:97 (1991). Also see Bird *et al.*, *Science* *242*:423 (1988), Ladner *et al.*, U.S. Patent No. 4,946,778, Pack *et al.*, *Bio/Technology* *11*:1271 (1993), and Sandhu, *supra*.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an

antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick *et al.*, *Methods: A Companion to Methods in Enzymology* 2:106 (1991); Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 166-179 (Cambridge University Press 1995); and Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.*, (eds.), pages 137-185 (Wiley-Liss, Inc. 1995).

Researchers have found that an anti-receptor monoclonal antibody can mimic the cognate ligand by binding with the ligand-binding domain of the receptor. For example, ligand-mimicking antibodies have been made that bind with a granulocyte-macrophage colony-stimulating factor receptor, a very low-density lipoprotein receptor, and a receptor encoded by the *c-erbB-2* gene. Von Feldt *et al.*, *Immunol. Res.* 13:96 (1994); Shawver *et al.*, *Cancer Res.* 54:1367 (1994); Pfistermueller *et al.*, *FEBS Lett.* 396:14 (1996). Antibodies that mimic JH can be screened, for example, using a competition assay with radiolabeled ligand, such as juvenile hormone, and JHR, as described below.

6. Use of a bHLH-PAS/JHR Protein to Screen for Juvenile Hormone Analogs and Antagonists

a. *In vitro* binding assays

Potential insecticides can be tested *in vitro* by determining the ability of a test compound to displace detectably-labeled JH (or labeled JH analog) from a recombinant bHLH-PAS/JHR. Assays designed to measure the binding of a ligand to a JH binding protein are well-established. See, for example, Ożyhar *et al.*,

Experientia 42:1276 (1986); Shemshedini and Wilson, *Insect Biochem.* 18:681 (1988); Shemshedini *et al.*, *J. Biol. Chem.* 265:1913 (1990); Shemshedini and Wilson, *Proc. Nat'l Acad. Sci. USA* 87:2072 (1990); Chang *et al.*, *Comp. Biochem. Physiol.* 99C:15 (1991); Glinka *et al.*, *Insect Biochem. Molec. Biol.* 25:775 (1995). However, the insecticide screening assay described herein uses a recombinantly-produced bHLH-PAS/JHR protein to test binding.

In one form of assay, a recombinant bHLH-PAS/JHR is incubated with labeled ligand, and a potential juvenile hormone-type insecticide is tested by measuring the ability of the compound to displace the labeled ligand bound to the recombinant receptor protein. Ligands, such as JH III or an analog, can be detectably labeled with a radiolabel, fluorescent label, a chemiluminescent label, or a bioluminescent label. Examples of suitable radioligands include ligands having one or more atoms enriched in a radioisotope, and ligands that are covalently coupled to a radioisotope label. Examples of radioisotopes that can be used to enrich ligands include ^{3}H and ^{14}C. Examples of radioisotopes that may be used to covalently label ligands include ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe, and ^{58}Se.

Suitable radiolabeled ligands include [^{3}H]10*R*-JH III and [^{3}H]methoprene. Radiolabeled JH III is commercially available. Radiolabeled ligands can also be obtained by chemical synthesis or biosynthesis, as described by Jennings *et al.*, "Labeled Compounds in Juvenile Hormone Research," in JUVENILE HORMONE BIOCHEMISTRY, Pratt *et al.* (eds.) pages 375-380 (Elsevier/North-Holland Biomedical Press 1981).

As an illustration, an insecticide screening assay is performed in a physiologically-compatible buffer such as 10 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, 10% glycerol and 100 μg/ml bovine serum albumin. The test

compound and labeled ligand (*i.e.*, JH or an analog) is dissolved in an alcohol, such as methanol, or in dimethylsulfoxide and diluted with assay buffer.

Recombinant bHLH-PAS/JHR is diluted with the assay buffer and incubated with [^{3}H]10*R*-JH III at room temperature for about thirty minutes. Two hundred microliters 200 μl of assay buffer is added to test tubes, followed by 100 μl of solution containing recombinant bHLH-PAS/JHR bound with the radiolabeled ligand (about 12,000 dpm per tube or 0.03 pmol). As a control, aliquots of JH III, about 0.023 pmol to about 3 pmol, are added to parallel series of tubes. After mixing the solutions, the tubes are allowed to incubate for thirty minutes at room temperature. The skilled artisan will recognize that this assay also may be carried out with JHI, JHII and methoprene as binding competitors, at concentrations of 0.023 pmol to 30 pmol. This serves as a measure of the specificity and apparent affinity of the bHLH-PAS/JHR for binding JHIII.

Hydroxyapatite (HAP) is used to separate bound radiolabeled ligand from free radiolabeled ligand, according to the procedure of Roberts *et al.*, *Molec. Cell. Endocrinol.* *31*:53 (1983), or a modification of the Roberts procedure. Briefly, 0.5 ml of 5% HAP (DNA-Grade HTP; Bio-Rad) suspension in HAP buffer (10 mM Tris-HCl [pH 8.0], 10 mM KH_2PO_4, 1 mM EDTA) was added. After gently vortexing the tubes, the solutions are incubated at 30 minutes at room temperature, and then filtered through glass fiber filters (*e.g.*, Whatman 934-AH). The filters are rinsed four times with HAP buffer, placed in vials, and dried at 100°C for about ten minutes. Scintillation fluid is then added to the tubes and allowed to incubate overnight before counting. Nonspecific binding is measured in the presence of 100-fold excess of unlabeled ligand (*i.e.*, JH III in this example). The results of the competition binding assay

are analyzed using established methods, such as Scatchard analysis. Scatchard, *Ann. N.Y. Acad Sci. 51*:660 (1949).

Although the insecticide screening assay has been described in considerable detail, it will be obvious to the practitioner in the art that modifications can be practiced within the scope of this invention. For example, the pH and the solution components can be modified, the time and temperature values may be varied, and modifications can be made in the form and material (*e.g.*, glass, plastic, *etc.*) of vessels used to perform the assay. Moreover, receptor-ligand complexes can be separated from unbound ligand by using centrifugation instead of filtering, or by using a suspension of charcoal-dextran instead of HAP. Alternatively, a "scintillation proximity assay" (SPA) can be used for screening insecticides. This technique provides sensitivity and minimal manipulation. The SPA involves the use of solid scintillant beads that emit photons when in proximity to a radioligand. Bosworth *et al. Nature* 341:167 (1989). Attachment of binding protein to the beads obviates the need for separating bound from free radioligand because the signal emitted by the free radioligand is quenched by aqueous surroundings. Only radioligand that is bound to its receptor will generate a signal. Amersham sells a variety of beads, such as beads coated with lectins (*e.g.*, ConA, wheat germ agglutinin, and lecithin) that will bind receptor protein.

In one variation of the insecticide screening assay, recombinant bHLH-PAS/JHR protein is preincubated with test compound, and then incubated with a labeled photoaffinity analog of juvenile hormone, such as epoxy farnesyl diazoacetate. In this assay, a test compound that binds with the bHLH-PAS/JHR protein will protect the protein from the photoaffinity label. Techniques for photoaffinity labeling of JH binding proteins are

described, for example, by Shemshedini *et al.*, *J. Biol. Chem.* *265*:1913 (1990), and by Prestwich *et al.*, "Hot JH: Using Radioligands and Photoaffinity Labels to Decipher the Molecular Action of Juvenile Hormone," in INSECT JUVENILE HORMONE RESEARCH: FUNDAMENTAL AND APPLIED APPROACHES, pages 247-256 (INRA 1992).

In a second variation of the insecticide screening assay, recombinant bHLH-PAS/JHR protein is incubated with a detectably labeled test compound, such as a radiolabeled test compound. The objective of this assay is to measure the binding of the test compound to the bHLH-PAS/JHR.

In a third variation of the insecticide screening assay, a first recombinant bHLH-PAS/JHR protein and a second heterodimeric partner of bHLH-PAS/JHR are incubated with a labeled test compound. The objective of this assay is to measure the binding of the test compound to the bHLH-PAS/JHR--heterodimeric partner complex.

According to another approach to insecticide screening, antibodies or antibody fragments are used that mimic JH by binding to the ligand-binding domain of the bHLH-PAS/JHR. Competition assays can be performed in which either the antibody (or antibody fragment) or the test compound is detectably labeled. The production of such antibody ligand mimics is discussed above.

The skilled artisan will recognize that compounds that bind a bHLH-PAS/JHR protein, identified with the above-described *in vitro* assays, may be agonists or antagonists. The characterization of a compound as agonist or antagonist is carried out using the *in vivo* assays described below.

b. *In vivo* binding assays

In addition to such *in vitro* assays, recombinant bHLH-PAS/JHRs can be used to screen insecticides in *in vivo* systems. As an illustration, Arnold *et al.*,

Environ. Health Perspect. 104:544 (1996), describe a screening assay for xenoestrogens, such as o,p'-DDT, in which transfected yeast cells express the human estrogen receptor and contain a *LacZ* gene under the control of two estrogen response elements.

A suitable *in vivo* assay for screening insecticides comprised incubating test compounds with yeast strains engineered to contain a functionally expressed *Met*-JHR. Functional expression can be achieved by using a one-hybrid or two-hybrid system. Luban *et al. Curr. Opin. Biotechnol.*, 6:59 (1995); Rowlands *et al. Pharmacol. & Toxicol.* 76:328 (1995); Yamaguchi *et al. Biochem. Pharmacol.* 50(8):1295 (1995).

Suitable vectors for *in vivo* assays include, but are not limited to, yeast, mammalian, and insect expression vectors. See, for example, Mak *et al. J. Biol. Chem.* 264:21653 (1989) and McDonnell *et al. Mol Cell. Biol.* 9:3519 (1989). In yeast, recombinant protein is expressed as an in-frame fusion to ubiquitin and an endogenous yeast ubiquitinase cleaves the fusion protein to release mature recombinant protein. Promoter such as TDH (constitutive) and CUP1 (copper inducible) may be used. In mammalian cells, a two-hybrid system has been used to test candidate transcriptional activators. Boudjelal *et al. Genes & Dev.* 11:2502 (1997). Boudjelal expressed fusions of the GAL4 amino terminus (147 aa encode DNA BD, dimerization domain, and nuclear localization signal) or the estrogen receptor DNA BD, and candidate TADs. These constructs were expressed in COS cells containing a reporter construct.

Two-Hybrid Assays -- Luban *et al. Curr. Opin. Biotechnol.*, 6:59 (1995) describes a two hybrid system. Any pair of proteins that interact with each other can be used to bring together separate DNA-binding (DNA BD) and transactivation domains (TAD) to reconstitute a transcriptional activator. The two-hybrid system is used

to study protein-protein interactions. Thus, in a typical application, two DNA constructs are used: (1) encodes protein X fused to a DNA BD (*e.g.*, from GAL4) (hybrid #1) and (2) encodes protein Y is fused to an AD (*e.g.*, from GAL4) (hybrid #2). A third construct is included which comprises a reporter gene under the control of a corresponding response element (*e.g.*, from GAL4). If proteins X and Y functionally interact, they will then bind to the GAL4 response element and stimulate expression of the reporter gene, by interacting with the GAL4 response element. If the reporter gene is not expressed, then a third (heteromultimeric partner) may be required.

In one version of a two-hybrid system, (1) *Met*-JHR is fused to one of the two-hybrid system proteins (DNA BP or a ADP) and (2) the heterodimeric partner of *Met*-JHR (which can be identified by a two-hybrid screen) is fused to the other protein of the two-hybrid system. Expression of the reporter gene, fused to the corresponding response element, will be effected if JH or a JH analog promotes the association between the *Met*-JHR protein and its heterodimeric receptor. See Ozenberger *et al. Molecular Endocrinology*, 9: 1321 (1995) and Lao *et al. Mol. Endocrin.* 11:366 (1997).

In another two hybrid system, a bHLH-PAS/JHR is expressed as a fusion protein with a DNA BP, a second plasmid expresses the bHLH-PAS/JHR protein as a fusion protein with an AD, and an appropriate reporter gene is also transfected into the cell. Treatment with JH would brings the two monomers to form an active homodimeric complex.

<u>One-Hybrid Assays</u> -- In a one-hybrid system, there is only one hybrid protein comprising a candidate binding protein and all or part of a protein having a DNA BD and an AD. One variation of the 1-hybrid system involves the use of a small molecular weight molecule, such as Dioxin.

Dioxin binds the AhR (also called the dioxin receptor) and will activate a GAL4-AhR construct. See Rowlands *et al. Pharmacol. & Toxicol.* 76:328 (1995). Rowlands' GAL4-AhR construct was "constitutively" active in yeast cells. In other words, the construct expressed a protein that turned on transcription of a *LacZ* reporter gene (*LacZ* fused to a GAL4 response element) in the absence of ligand. However, the addition of dioxin enhanced transactivation. Additionally, Whitelaw fused the DNA BD of the glucocorticoid receptor to the AhR and transformed CHO mammalian cells with this construct. *Mol. Cell. Biol.* 14:8343 (1991). The addition of dioxin to these cells induced expression of a reporter gene linked to a glucocorticoid response element. If a recombinant protein X comprises a TAD, then fusing X to a DNA BD may produce a constitutively active construct (*e.g.*, GAL4 DNA BD/X-AD). Such a construct can be used to screen for antagonists. The addition of an antagonist will inhibit expression of a reporter gene linked to a GAL4 response element.

In another assay, the GAL4 DNA BD/X-AD is trascriptionally silent unless it is activated by another component. In a one-hybrid system, the heteromultimeric partner is consitutively expressed from another plasmid. A reporter plasmid contains a GAL4 response element linked to a reporter gene. Antagonists are screened for their ability to inhibit expression of the reporter gene.

Thus in another embodiment of the one-hybrid system, a bHLH-PAS/JHR is fused to the GAL4 DNA BD and an appropriate reporter plasmid is also transfected into the cell (*e.g.*, GAL4 response element fused to a reporter gene). Treatment of the cell with JH results in induction of reporter gene expression if JH specifically binds bHLH-PAS/JHR.

If a heterodimeric partner is necessary for activation, it is expressed from a separate plasmid. The bHLH-PAS/JHR-GAL4 DNA BD fusion protein forms a heterodimer with the partner and upon addition of JH, the complex would activate transcription.

One- and Two-hybrid system components -- Truncated forms of *Met*-JHR and chimeras of *Met*-JHR with other bHLH-PAS proteins can be made and expressed in the one- or two-hybrid system. For example, removal of the bHLH domains before making the fusions may be desirable. The skilled artisan will recognize that functional fragments, as described above, are suitable for use in the *in vivo* assays. For example, a DNA BD of a bHLH-PAS/JHR protein may be fused to the AD of a second protein. This allows screening compounds that interfere with DNA binding by a bHLH-PAS/JHR protein. Furthermore, truncated forms of heterodimeric partners may also be suitable for use in the above-described assays.

Suitable heterodimeric partners for a bHLH-PAS/JHR include, but are not limited to, DARNT (a bHLH-PAS protein) and ultraspiracle, a nuclear orphan/steroid receptor. *Drosophila* has at least three bHLH-PAS proteins, *Sim*, *Trh*, and DARNT. Zelzer *et al*. presented evidence that *Sim* and *Trh* each form heterodimenrs with DARNT. *Genes & Dev*. 11:2079 (1997).

Suitable reporter polypeptide for use in the above-described assays include, but are not limited to, ß-galactosidase derived from *E. coli* (LacZ); genes conferring sensitivity to a chemical such as CYH2, cycloheximide sensitivity, and CAN1, canavanine sensitivity, arginine permease derived from *S. cerevisiae* (CAN1). The expression of these reporter genes in the presence of the toxic substrate (cycloheximide, etc.) results in the suppression of cell growth. This is convenient for looking for antagonist compounds, *i.e.*, compounds that permit cell growth. This is referred to

as a "rescue screen." Other suitable reporter polypeptide include those involved in nucleoside and amino acid metabolism, such as the products of the URA3, LEU2, LYS2, HIS3, HIS4, TRP1, and ARG4 gene; polypeptide that confer resistance to drugs such as hygromycin, tunicamycin, cyclohexamide, and neomycin; and green fluroescence protein (GFP). Guthrie *et al. Meth. Enzymol.* vol. 194 (1991); Prasher, *Trends Gen.* 11:320 (1995). Detection of reporter gene expression is achieved using methods that are well-known to the skilled artisan.

Suitable transcription activation proteins include, but are not limited to, Gal4, Gcn4, Hap1, Ard1, Swi5, Ste12, Mem1, Yap1, Ace1, Ppr1, Arg81, Lac9, Qa1F, VP16, LexA, non-mammalian nuclear receptors (*e.g.*, ecdysone) or mammalian nuclear receptors (*e.g.*, estrogen, androgen, glucocorticoids, mineralocorticoids, retinoic acid and progesterone. See also Picard *et al. Gene* 86:257 (1990)

Suitable TADs include, but are not limited to, those from GAL4, Gcn4 or Adr1. A DNA binding protein domain can be substituted for the DNA binding domain of the transactivational activation protein, if the recognition sequences operatively associated with the reporter gene are correspondingly engineered. For example, non-yeast DNA binding proteins are mammalian steroid receptors and bacterial LexA. See Wilson *et al. Science* 252:1296 (1990).

7. Isolation of Additional Juvenile Hormone Receptor Genes

The nucleotide sequences of the *Met* JHR gene and antibodies to the receptor provide a means to isolate additional bHLH-PAS/JHR genes from other insects. Such genes can encode receptors specific for JH molecules of various species, including insects of the orders *Coleoptera* (*e.g.*,root worm, cigarette beetle, potato

beetle, cotton boll weevil, and grain pests), *Siphonaptera* (fleas), *Orthoptera* (roaches), *Isoptera* (termites), *Thysanoptera* (thrips), *Lepidoptera* (butterflies and moths), *Hemiptera* (junebugs), *Hymenoptera* (ants), and *Diptera* (flies). In regard to the order *Hemiptera*, insects of the suborders *Heteroptera* (*e.g.*, true bugs, stink bugs, and tarnished plant bug) and *Homoptera* (*e.g.*, aphids, white flies, scale insects, and leaf hoppers) are of particular interest. Examples of suitable insects in the order *Lepidoptera* include species in the genera *Spodoptera* (*e.g. S. lieeralis*, *S. exigua*, and *S. frugiperta*), *Mamestra* (*e.g.*, *M. brassica*, the cabbage moth), *Plutella* (*e.g.*, *P. xylostella*, the diamondback moth), *Pieris* (*e.g.*, *P. rapae*, the cabbage butterfly), *Heliothis* (*e.g.*, H. zea, the corn earworm, as well as the bollworm and tobacco budworm). Suitable insects of the order *Diptera* include flies, such as horn fly, fruit fly, screwworm fly, blow fly, mosquito, mediterranean fruit fly, biting midge, black fly, horse fly, deer fly, leaf miner, housefly, bot fly and warble fly.

For example, bHLH-PAS/JHR genes can be isolated from agricultural pests, including insects of the genera *Ceroplastes* (*e.g.*, *Ceroplastes floridensis*, Florida wax scale), *Saissetia* (*e.g.*, *Saissetia oleae*, black scale), *Aonidiella* (*e.g.*, *Aonidiella aurantii*, California red scale), *Chrysomphalus* (*e.g.*, *Chrysomphalus aonidum*, Florida red scale), *Dacus* (*e.g.*, *Dacus oleae*, olive fruit fly), *Megaselia* (*e.g.*, *Megaselia alterata*, mushroom hump-backed fly), *Lycoriella* (*e.g.*, *Lycoriella auripila*, mushroom fungus gnat), *Schistocerca* (*e.g.*, *Schistocerca gregaria*, desert locust), *Diatraea* (*e.g.*, *Diatraea grandiosella*, Southwestern corn borer), *Achoea* (*e.g.*, *Achoea janata*, castor semi-looper), *Spodoptera* (*e.g.*, *Spodoptera littoralis*, Egyptian cotton leaf worm), and *Mamestra* (*e.g.*, *Mamestra brassicae*, cabbage armyworm).

bHLH-PAS/JHR genes can also be isolated from stored product pests, including insects of the genera *Tribolium* (*e.g.*, *T. castaneum* [red flour beetle], *T. confusum* [confused flour beetle]), *Tenebrio* (*e.g.*, *Tenebrio molitor*, yellow mealworm), *Rhyzopertha* (*e.g.*, *Ryzopertha dominica*, lesser grain borer), *Sitophilus* (*e.g.*, *Sitophilus oryzae*, rice weevil), *Oryzaephilus* (*e.g.*, *Oryzaephilus surinamensis*, saw-toothed grain beetle), *Plodia* (*e.g.*, *Plodia interpunctella*, Indian meal moth), and *Sitotroga* (*e.g.*, *Sitotroga cerealella*, angoumois grain moth). Suitable sources for bHLH-PAS/JHR genes also include forest insect pests, such as *Choristoneura* (*e.g.*, *Lamentria dispar* (Gypsy moth), C. fumiferana [Eastern spruce budworm], *C. occidentalis* [Western spruce budworm]), *Lambdina* (*e.g.*, *Lambdina fiscellaria*, Eastern hemlock looper), and *Dendroctonus* (*e.g.*, *D. pseudotsugae* [Douglas fir beetle], *D. frontalis* [Southern pine beetle]).

bHLH-PAS/JHR genes can also be isolated from insects considered to be of public and veterinary significance, as well as insects simply considered as a nuisance. Examples include mosquito species (*e.g.*, *Culex pipiens*, *Aedes aegypti*, *Anopheles albimanus*, as well as *A. albopictus* (tiger mosquito) and *C. tarsalis* (swamp marsh mosquito)), blackfly species (*e.g.*, *Simulium venustum*, *Prosimulium mixtum*), dog ticks (*e.g.*, *Ctenocephalides canis* and *Dermacentor andersoni*), cattle ticks (*e.g.*, *Boophilus microplus*), lice (insects of the orders *Mallophaga* or *Anoplura*), as well as insects of the genera *Haematobia* (*e.g.*, *Haematobia irritans*, horn fly), *Musca* (*e.g.*, *M. autumnalis* [face fly], *M. domestica* [house fly]), *Glossina* (*e.g.*, *G. palpalis*, *G. morsitanstsete* [tsetse fly]), *Melophagus* (*e.g.*, *Melophagus ovinus*, sheep ked), *Monomorium* (*e.g.*, *Monomorium pharaonis*, Pharaoh's ant), *Solenopsis* (*e.g.*, *Solenopsis invicta*, imported fire

ant), and *Reticulitermes* (*e.g., Reticulitermes flavipes*, Eastern subterranean termite).

Lepidopteran species of interest include, but are not limited to: other Heliothia species, such as the American bollworm, H. armigera and the bollworm, H. punctigera; lepidopteran species of the genus Spodoptera, e.g., the Egyptian cotton leafworm, s. litteralia, the best armyworm, s. exigа; the fall armyworm, s. fruciperda; the cutworm, B. litura, the rice swarming caterpillar, S. muritania and the southern armyworm, S. eridania; and other miscellaneous lepidopterans, e.g., the pink bollworm, Pectinophora gossypiella; the spiny bollworm, Barius insulana, the cotton leafworm, Alabama argillacea; the leaf perforator, Bucculatris thurberiella; the tomato fruitworm, Helicoverpa sea; the diamondback moth, Plutella xylostella; the cabbage looper, Trichoplasia ni; the imported cabbageworms Hellula undalis and Hellula rogatalis; the black cutworm, Agrotis ipsilon; the corn earworm, Ostrinia nubalis; the tomato pinworm, Keiferea; lycopersicella; the tomato hornworm, Manduca sexta and Manduca guinquemaculata; the velvet bean caterpillar, Anticarsia_gemmatalis; the green oliveworm, Plathypena scabra; the soybean looper, Pseudoplusia includens; the saltmarsh caterpillar, Estigmene acrea; the leaf miner, Epinotia meritana; the codling moth, cydia pomonella; the oblique banded leafroller, Choristoneura rosaceana; grape berry moth, Lobesia botrans; currant tortrix, Pandemis cerasana; spotted tentiform leafminer, Phyllonocytes blancardella; grape leafroller spargano-this pilleriana; tufted bud apple moth, Platynota idacusalis; red banded leafroller, Argyrotaenea velutinana; oriental fruit moth, Grapholitha molesta; Southwestern corn borer, Diatrea grandiosella; rice leafrollers, Chaphalocrocis medinalis, Marasmia exigua and Marasmia patnalis; striped borer, Chilo suppressalis; dark headed stem-borer, Chilo polychrysis; yellow stem borer, Scirophaga incatulae;

white stem borer, Scirophaga innotata; and pink stem borer, Sesamia inferens.

Non-lepidopteran species include the Colorado potato beetle Leptinotarsa decimlineator, the boll weevil, Anthonomus grandis; the Southern corn rootworm, Diabrotica undecimpunctata; the Japanese beetle, Popillia japonica; plum curculio, Conotrachelus nenuphar; brown planthopper, Nilaparvata lugens; green leafhopper, Naphotettix virescens; potato leafhopper, Empoasca abrupta; cotton aphid, Aphis gossypii; green peach aphid, Myzus persicae; sweetpotato whitefly, Bemisia tabaci; imported fireant, Solenopsis invicta; thrips, e.g., Thrips palini; pear psylla, Psylla pyri; two-spotted spider mite, Tetranychus urticae; carmine mite. Tetranychus cinnabarinus; citrus rus mite, Phyllocoptruta oleivora; German cockroach, Blatella germanica; cat flea, Ctenocephatides felia; yellow fever mosquito, Aedes aegypti; and salt marsh mosquito, Aedes sollioitans.

In one screening approach, polynucleotide molecules having nucleotide sequences disclosed herein can be used to screen genomic or cDNA libraries. Screening can be performed with *Met* gene polynucleotides that are either DNA or RNA molecules, using standard techniques. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, pages 6-1 to 6-11 (John Wiley & Sons, Inc. 1995). Insect genomic and cDNA libraries can be prepared using well-known methods. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, pages 5-1 to 5-6 (John Wiley & Sons, Inc. 1995).

Additional bHLH-PAS/JHR genes can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the *Met* JHR nucleotide sequences described herein. General methods for screening libraries with PCR are provided by, for example, Yu *et al.*, "Use of the Polymerase Chain Reaction to Screen Phage Libraries," in

METHODS IN MOLECULAR BIOLOGY, Vol. 15: PCR PROTOCOLS: CURRENT METHODS AND APPLICATIONS, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in METHODS IN MOLECULAR BIOLOGY, Vol. 15: PCR PROTOCOLS: CURRENT METHODS AND APPLICATIONS, White (ed.), pages 317-337 (Humana Press, Inc. 1993).

Anti-*Met* JHR antibodies can also be used to isolate DNA sequences that encode bHLH-PAS/JHRs from cDNA libraries. For example, the antibodies can be used to screen λgt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation. See, for example, Ausubel *et al.* (eds.), SHORT PROTOCOLS IN MOLECULAR BIOLOGY, 3rd Edition, pages 6-12 to 6-16 (John Wiley & Sons, Inc. 1995); and Margolis *et al.*, "Screening λ expression libraries with antibody and protein probes," in DNA CLONING 2: EXPRESSION SYSTEMS, 2nd Edition, Glover *et al.* (eds.), pages 1-14 (Oxford University Press 1995).

8. RFLP Analysis -- Screening Populations for Resistance

Polynucleotides that encode bHLH-PAS/JHRs can be used to monitor an insect population for resistance to JH analog insecticides. For example, polynucleotides encoding *Drosophila* bHLH-PAS/JHRs from methoprene-sensitive and methoprene-resistant insects can be used to screen a pest insect population for an increase in methoprene resistance by restriction fragment length polymorphism (RFLP) analysis. Similarly, Dong *et al. Insect Biochem. Mol. Biol. 24*:647 (1994), have used RFLP analysis to examine the presence of knockdown resistance to pyrethroid insecticides in cockroach populations, Williamson *et al., Mol. Gen. Genet. 240*:17 (1993), identified RFLPs associated with resistance to DDT and

pyrethroid insecticides in the housefly, while Severini *et al.*, *J. Med. Entomol.* *31*:496 (1994), used RFLP analysis to examine a possible increase in the frequency of an insecticide resistance gene (esterase B) in populations of the disease-bearing mosquito, *Culex pipiens*. RFLP analysis has also been used to examine populations of various mosquito species and screwworms. Severson *et al.*, *Am. J. Trop. Hyg.* *50*:425 (1994); Taylor *et al.*, *Med. Vet. Entomol.* *10*:63 (1996).

In an analogous approach, researchers have used RFLP analysis to differentiate between non-aggressive and aggressive strains of fungi and bacteria that are plant pathogens. Koch *et al.*, *Mol. Plant-Microbe Inter.* *4*:341 (1991); Graham *et al.*, *PhytoPathology* *80*:829 (1990). Also, see generally, Miller *et al.*, "Diagnostic Techniques for Plant Pathogens," in BIOTECHNOLOGY IN PLANT DISEASE CONTROL, Chet (ed.), pages 321-339 (Wiley-Liss, Inc. 1993), Zilberstein *et al.*, "Application of DNA Fingerprinting for Detecting Genetical Variation Among Isolates of the Wheat Pathogen *Mycosphaerella graminicola*," in BIOTECHNOLOGY IN PLANT DISEASE CONTROL, Chet (ed.), pages 341-353 (Wiley-Liss, Inc. 1993), and Honeycutt *et al.*, "Application of the Polymerase Chain Reaction to the Detection of Plant Pathogens," in THE IMPACT OF PLANT MOLECULAR GENETICS, Sobral (ed.), pages 187-201 (Birkäuser 1996).

The identification of a genetic variant associated with juvenile hormone analog resistance (e.g., methoprene resistance) provides several avenues for testing to monitor the occurrence and frequency of insecticide resistance in a population at a very early stage when the frequency may be very low and/or difficult to detect by standard bioassays. This early detection facilitates informed judgments in the application of the relevant insecticide. For example, the gene *Met* encoding the juvenile hormone receptor (*e.g.*, Met^{43}) provides the basis

for RFLP analysis of an insect population to identify the presence of the resistance trait in a given population. T. Wilson J. Econ. Ent. 86: 645-651 (1993).

Detection of the unique DNA associated with a resistance allele is diagnostic for the appearance of the resistance trait in the population sampled. This is determined by digesting genomic DNA collected from individuals of the target population in question with selected restriction enzyme(s) followed by probing a Southern blot with a detectably labelled DNA sequence that identifies a particular resistant trait, or a diagnostic portion thereof. By "diagnostic portion" thereof is meant any fragment of DNA from a bHLH-PAS JHR DNA sequence which differs sufficiently in sequence from the corresponding portion of the susceptible DNA sequence so as to be detectable in a Southern blot. A "diagnostic portion" may also be a unique DNA sequence genetically linked within one map unit of the trait that can be detected in a Southern blot analysis. DNA sequences flanking the resistance gene, as well as intervening sequences (introns) are particularly suited for identifying unique diagnostic RFLPs.

For example, the methoprene resistant alleles, Met^{KI7} and Met^{A3}, contain 1300 kb P-element insertions within the 1200bp Bam H1 fragment immediately upstream from the Met^{+} gene transcriptional start site. In this technique, DNA from several individuals in the target population is digested with an appropriate restriction enzyme, and size separated by gel electrophoresis. The gel, or a blot derived therefrom, is then probed with labelled DNA, using either the whole gene or a diagnostic fragment. If there are both resistant and sensitive alleles within an individual in the population, there will appear on the gel at least two different sized restriction fragments. Segments each fragment will hybridize with the bHLH-PAS/JHR gene probe.

In this manner, large numbers of individuals in the population can be sampled, and the relative abundance of an allele can be determined. Identification of the specific DNA fragment associated with resistance, whether by Southern or RFLP analysis, will always be diagnostic.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

Example 1

To isolate the *Met* JHR gene, genomic libraries were constructed from flies carrying either of two P-element alleles of *Met*, Met^{A3} and Met^{KI7}. These alleles were recovered in separate screens from methoprene-resistant flies. Wilson *et al. Molecular Mech. of Insecticide Resistance* (Am. Chem. Soc. Symp.) 505:99 (1992). Each allele conferred resistance to both the toxic and morphogenetic effects of JH and methoprene, and susceptible revertants could be recovered by standard genetic means. A 50 kilobase region surrounding the P-element insertion site was cloned from each library, as described by Turner and Wilson, *Arch. Insect Biochem. Biophys.* *30*:133 (1995).

DNA sequencing and analysis of the genomic region located within one kilobase of the insertion sites revealed an open reading frame (ORF) located 273 base pairs from the insertion site in the Met_{A3} allele and 424 base pairs in the Met^{KI7} allele. See Figure 1. Transcription of this ORF occurs away from the P-element, suggesting possible interruption of transcription by the P-element, as has been found with other P-element mutations in *Drosophila*. Searles *et al.*, *Mol. Cell Biol.* *6*:3312 (1986); Kelley *et al.*, *Mol. Cell. Biol.* 7:1545 (1987).

P-element mediated germline transformation was carried out with DNA fragments isolated from phage clones derived from a wild-type genomic library. Genomic fragments were isolated following restriction enzyme digestions of phage obtained from the iso-1 strain genomic library. (Tamkun *et al. Proc. Natl. Acad. Sci. USA* 81:5140 (1984)). The locations of the restriction sites for each fragment are shown in Figure 1. Fragments were either subcloned into Bluescript (Stratagene Co., Ca.), then excised with an *Eco*RI-*Not*I double digest and subcloned into the pCaSpeR 4 transformation vector (Thummel *et al. Drosophila Inform. Serv.* 71:150 (1992)), or were subcloned directly into the pCaSpeR 4 vector. Purified plasmids together with pp25.1wc transformation "helper" DNA ina ratio of 2-3:1 were injected into dechorionated *ywMet*^{3} embryos as described. (A. Spradling in Drosophila -- A Practical Approach (D.B. Roberts, Ed. IRL, Oxford, 1986, page 175). Go progeny were individually crossed with *y w Met*^{3}, and transformants recognized by restoration of eye color ranging from light orange to red. For each DNA fragment, 3-5 transformants from separate Go females were recovered and their progeny tested for methoprene resistance.

Files carrying *Met*^{3}, a strong EMS-induced allele, were transformed with fragments shown in Figure 1. Progeny of transformants were tested on three diagnostic amounts of methoprene as described in Wilson, *Arch. Insect Biochem. Physiol.* 32:641 (1996)). Resistant and susceptible animals were distinguished on the basis of survival as well as the presence of normal morphology of sternal bristle patterns and male genetalia.

When transformants with fragments St-X and K-H were tested for methoprene resistance, the level of resistance was undiminished compared with that of *Met*^{3}, indicating no rescue of the mutant phenotype. However, when *Met*^{3} was transformed with the 6.234 Kb St-H fragment,

resistance was lost, indicating that a functional *Met* sequence is contained in this sequence. The DNA region contained in fragment St-H corresponds well with the size and location of the transcripts.

The nucleotide sequence of the 6.234 Kb St-H fragment of Figure 1 is shown in Figure 2 (SEQ ID NO:1). The St-H fragment comprises an intron (lower case letters) and an open reading frame from base no. 1514 to base no. 3732. Also shown in the Figure are the first (no. 1292) and last (no. 4301) bases of the genomic *Met*-JHR sequence in Figure 3.

The St-H fragment was cloned into the *Sma*I site of the Bluescript(ks) vector, and is designated pSt-H. Vector pSt-H was deposited at the American Type Culture Collection, in Bethesda, Maryland, on November 13, 1997. The present invention includes a polynucleotide having the nucleotide sequence of the St-H fragment in vector pSt-H.

Example 2

A DNA probe to the ORF described above failed to identify any transcript(s) on a Northern blot of RNA from a methoprene-susceptible Oregon-RC late third-instar larvae, but a more sensitive RNA probe recognized a transcript of approximately 5.5 kilobases. Total RNA was isolated with TriReagent (Molecular Research Center, Inc., Ohio) from staged animals. Each lane was loaded with 40 mg of total RNA, subjected to denaturing gel electrophoresis on a formaldehyde-agarose gel, and blotted onto Hybond-N membrane. Following cross-linking, membranes were prehybridized in a solution containing 5X SSPE, 5X Denhardt's, 0.5% SDS, 50% formamide, and 100 μg/ml yeast tRNA for about 5 to about 7 hours at about 65°C.

Membranes were then hybridized in the same solution at about 68°C for about 15 to about 17 hours with a [^{32}P]-UTP labeled riboprobe (Promega Co., WI) synthesized from

a fragment of the *Met*-JHR gene. This fragment extended from nucleotide 771 through 1102 of the open reading frame, where nucleotide #1 is designated the base A in the ATG codon that begins the open reading frame. This corresponds to base no. 1514 through base no. 1845 in Figure 2. The 771-1102 fragment was produced by PCR amplification from a genomic clone from the iso-1 phage containing the *Met* region. The amplified fragment was subcloned into a T-vector (Invitrogen, Ca.), linearized with *SST* II, and transcribed from the T7 promoter to produce a 331 bp antisense RNA molecule (the reverse transcript of the 771-1102 DNA fragment). The membranes were washed with 2X SSC + 0.1% SDS at about 22°C for about 20 minutes, followed by two washes with 0.1X SSC + 0.1% SDS at about 65° for about 15 minutes each. Each membrane was placed against X-ray film and subjected to autoradiography at about -70°C for 24 hours and developed. Control loading was evaluated by stripping the blot and reprobing with a [^{32}-P]-dCTP random-primed cDNA for the *ribosomal protein-49 gene* (*Rp 49*, O'Connell *et al. Nucl. Acid. Res.* 12:5495 (1984)).

As shown in Figure 8, the level of the 5.5 kb transcript was undiminished in three EMS-induced alleles of *Met* (*Met*, *Met*^{2}, and *Met*^{3}). The transcript was reduced in several alleles that were X-ray induced from methoprene-susceptible *vermillion* (v) flies (*Met*^{N6}, *Met*^{D29}, *Met*^{11}, *Met*^{27}, *Met*^{128}), especially *Met*^{27}, which appears as a null allele. *Met*^{A3} and *Met*^{K17} (P-element alleles) also showed a transcript that is approximately three kilobases larger than the 5.5 kilobase transcript, suggesting that transcriptional run-on of the 2.9 kilobase P-element is occurring in these flies. A similar transcriptional run-on observation has been seen with the P-element allele of *yellow* mutant.

The boundaries of the 5.5 kb transcript have not been precisely determined, but they have been inferred by RT-

PCR to include a transcriptional start site about 1,100 bp upstream of the ATG site and an end site about 2,200 bp from the stop codon of the ORF. See Figure 1.

As shown in Figure 9, a Northern analysis was carried out to determine the abundance and temporal appearance of *Met*-JHR transcripts. During the first half of embryonic development, a transcript of approximately 3.3 kilobases was detected in methoprene-susceptible Oregon-RC adult females. Total RNA was isolated from these females at various times during development. Each lane of the gel was loaded with with 40 mg of total RNA, and the blot was probed with the 331 bp *Met*-JHR riboprobe, followed by a DNA probe for the *Rp49* gene, as described above. Embryos were collected from overnight cultures and either frozen in liquid nitrogen or maintained at 25°C until the desired age (0-10 hours, 8-16 hours, 16-24 hours). Larvae were staged from timed embryo collections (2 days +/- 8 hrs;
3 days +/- 8 hrs; 4 days +/- 8 hrs; white prepupa). Pupas were staged from the white prepupal stage, which lasts about one hour (1 day, 2 day, 3 day, and 4 day). Adult males consistently show only the 5.5 Kb transcript. Females show both the 3.3 kb and the 5.5 kb transcript, and when fully gravid, show increased levels of the 3.3 kb transcript. The 3.3 Kb transcript is present only in embryos and adult ovary tissue. Additionally, since methoprene-sensitivity in fly development is found only in late larval-early pupal stage, the appearance of the 3.3 Kb transcript is not correlated with methoprene resistance.

Example 3

cDNA molecules corresponding to the region containing the *Met*-JHR ORF, as well as to the smaller (3.3 kb) transcript, were isolated as apparent full-length cDNAs from a *Drosophila* wild-type Canton-S ovary cDNA library and were sequenced to establish a relationship of the

transcript with the genomic nucleotide sequence. The probable transcription start site for this transcript begins 220 bp upstream from the start codon and the probably transcript ends 912 bp from the stop codon.

A comparison of the cDNA to the genomic sequence showed that the genomic ORF is 2.22 Kb and the cDNA ORF is 2.151 Kb. The difference between the two sequences is a 69 nucleotide intron, which corresponds to 23 codons, and does not change the open reading frame of the genomic and cDNA. The presence of the intron provides evidence for the possibility of alternatively spliced variants of *Met*-JHR and hence multiple isoform proteins of *Met*-JHR.

The longest single open reading frame in the cDNA in Figure 3 (SEQ ID NO:3) comprises a single open reading frame (ORF). The DNA sequence (CAAAATGGCA) (SEQ ID NO:13) surrounding this ATG of the ORF is in good agreement with a *Drosophila* translation start site consensus sequence. Cavener *Nucl. Acid. Res.* 15:1353 (1987).

The first genomic exon is from position 224 to position 1543 (1296 bases). This is followed by a 69 bp intron, and a second exon, which extends from position 1589 to 2443 (855 bases). The remainder of the *Met*-JHR gene is from 2443 to 3011 (568 bases). The total length of the nucleotide sequence provided for the genomic DNA is 3011 nucleotides (SEQ ID NO:2), and that of the cDNA is 3282 nucleotides (SEQ ID NO:3).

Comparison of the *Met*-JHR ORF and with sequences deposited in the Genbank database showed three regions of homology to members of a family of transcriptional activators known as the basic helix-loop-helix-Per-Arnt-*Sim* (bhlh-PAS) proteins. See Figure 6. Three vertebrate members of this family include the aromatic hydrocarbon receptor nuclear translocator (ARNT), muscle and brain ARNT-like protein 1 (BMAL-1), and the aromatic hydrocarbon receptor (AHR). Three *Drosophila* family

members include ARNT (DARNT), Trachealess (*Trh*) and Single-minded (*Sim*).

The ARNT and AHR proteins are involved as heterodimeric partners in binding a variety of environmental toxicants, including dioxin, and subsequently activating a variety of genes important in the degradation of these chemicals, such as the cytochrome P450 genes. Figure 6 indicates that *Met*-JHR is neither DARNT nor AHR. However, *Met*-JHR shares considerable homology to human AHR in the ligand binding region of AHR, which is amino acids 200-400 of AHR. Rowlands *et al. Crit. Rev. Toxicol.* 27:109 (1997). Another feature apparent from visual inspection of the *Met*-JHR sequence is that *Met*-JHR, like human ARH (HARH), has a high concentration of serine and threonine residues at its carboxyl terminus. This is the motif of a S/P/T transactivation domain, as noted above. In ARH, this domain has been shown to be a functional TAD.

These features support the hypothesis that the mechanism of action of *Met*-JHR is similar to AHR, *i.e.*, *Met*-JHR binds the JH ligand. In addition, the *Met*-JHR may heterodimerize to DARNT or a DARNT-like protein in order to bind a JH response element and mediate JH action. The bHLH domain has been shown to be involved in dimerization and DNA binding. Rowlands *et al. Critical Reviews in Toxicology*, 27: 109 (1997).

Met-JHR also contains the "LXXLL" (SEQ ID NO:14) motif which likens *Met* to steroid receptor co-activators. Although this motif is found in many proteins, it plays a significant role in proteins that interact with co-activators of steroid receptors. LXXLL (SEQ ID NO:14) also has been found in a bHLH-PAS protein that is a cofactor (ACTR) [Chen *et al. Cell* 90:569 (1997)] that is amplified in breast cancer-1 (AIBC). Anzick *et al. Science* 277:965 (1977). This bHLH-PAS protein (ACTR/AIBC) interacts with a steroid

receptor, and is part of the muti-protein complex that potentiates the signal from the steroid receptor ligand.

Met-JHR also has homology with *Single Minded (Sim)*, a neurogenic transcriptional factor that has been identified as a *Drosophila* bHLH-PAS family member. However, *Sim* has not been identified as a ligand-binding protein.

Example 4

A JHR *Met* nucleotide sequence was isolated from a cDNA library from *D. erecta*, *Met*-JHR-*erecta*. A reverse oligonucleotide primer was based on the 5' end of the PAS-A region of the *Drosophila Met*-JHR gene, TLMQLL. Using this primer, standard polymerase chain reaction (PCR) techniques were used to amplify DNA sequences from *D. erecta*. The amplified DNA was subcloned into a plasmid and sequenced. The sequence obtained from *D. erecta* includes 232 nucleotides from the N-terminal portion of the *Met-erecta*-JHR ORF. As shown in Figure 5, there is high homology between the two genes, suggesting that the Met^{+} gene may be conserved throughout *Drosophila* and the order Diptera.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention

pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference in its entirety.

What Is Claimed Is:

1. An isolated polynucleotide that encodes a bHLH-PAS polypeptide that is involved in binding juvenile hormone III.

2. An isolated polynucleotide as claimed in claim 1, wherein said polynucleotide hybridizes under stringent conditions with a polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:6.

3. An isolated polynucleotide as claimed in claim 1, wherein said polynucleotide hybridizes under stringent conditions with a polynucleotide that encodes a polypeptide having the amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:5.

4. An isolated polynucleotide as claimed in claim 1, wherein said polynucleotide hybridizes under stringent conditions with a riboprobe that is the reverse transcript of a polynucleotide having the sequence of nucleotide 1514 through 1845 of SEQ ID NO:1.

5. An isolated polynucleotide as claimed in claim 1, wherein said polynucleotide hybridizes with a riboprobe that is the reverse transcript of a polynucleotide having the sequence of nucleotide 1514 through 1845 of SEQ ID NO:1, wherein said hybridization is carried out in 5X SSPE, 5X Denhardt's, 0.5% SDS, 50% formamide, and 100 μg/ml yeast tRNA for about 15 to about 17 hours at about 68°C.

6. An isolated polynucleotide of claim 1, wherein said insect is selected from the group consisting of

Coleoptera, Siphonoptera, Orthoptera, Thysanoptera, Lepidoptera, Hemiptera, and Diptera.

7. An isolated polynucleotide of claim 1, wherein said insect is a member of the order *Diptera* selected from the group consisting of horn fly, fruit fly, screwworm fly, blow fly, mosquito, mediterranean fruit fly, biting midge, black fly, horse fly, deer fly, stable fly, leaf miner, housefly, bot fly, warble fly, tiger mosquito, swamp marsh mosquito, *Culex pipieus*, *Aedes aegypti*, and *Anopheles albopictus*.

8. An isolated polynucleotide of claim 7, wherein said polynucleotide has been isolated from a fruit fly.

9. An isolated polynucleotide of claim 8, wherein said polynucleotide has a nucleotide sequence that encodes a polypeptide having an amino acid sequence selected from the group consisting of SEQ ID NO:3 and SEQ ID NO:4.

10. An isolated polynucleotide of claim 9, wherein said polynucleotide has a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

11. An expression vector comprising the isolated polynucleotide of claim 1.

12. A cultured host cell comprising the expression vector of claim 11.

13. A host cell of claim 12, wherein said host cell is selected from the group consisting of bacterial cell, yeast cell, insect cell and mammalian cell.

14. A method of producing a polypeptide, said method comprising the steps of:

(a) culturing a host cell comprising the expression vector of claim 11, wherein said cultured host cell expresses said bHLH-PAS polypeptide, and

(b) isolating said polypeptide from said cultured host cell.

15. An isolated polypeptide selected from the group consisting of:

(a) a conservative amino acid variant of SEQ ID NO:3,

(b) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:3,

(c) a polypeptide having an amino acid sequence of SEQ ID NO:3,

(d) a conservative amino acid variant of SEQ ID NO:4,

(e) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:4,

(f) a polypeptide having an amino acid sequence of SEQ ID NO:4, and

(g) a *Met*-JHR alternatively-spliced isoform.

16. The isolated polypeptide of claim 15, wherein said conservative amino acid variant is a polypeptide having an amino acid sequence that differs from the amino acid sequence of SEQ ID NO:3 by containing at least one amino acid substitution selected from the group consisting of (1) the substitution of an alkyl amino acid for an alkyl amino acid in SEQ ID NO:3, (2) the substitution of an aromatic amino acid for an aromatic amino acid in SEQ ID NO:3, (3) the substitution of a sulfur-containing amino acid for a sulfur-containing amino acid in SEQ ID NO:3, (4) the substitution of a hydroxy-containing amino acid for a hydroxy-containing

amino acid in SEQ ID NO:3, (5) the substitution of an acidic amino acid for an acidic amino acid in SEQ ID NO:3, (6) the substitution of a basic amino acid for a basic amino acid in SEQ ID NO:3, and (7) the substitution of a dibasic monocarboxylic amino acid for a dibasic monocarboxylic amino acid in SEQ ID NO:3.

17. The isolated polypeptide of claim 15, wherein said conservative amino acid variant is a polypeptide having an amino acid sequence that differs from the amino acid sequence of SEQ ID NO:4 by containing at least one amino acid substitution selected from the group consisting of (1) the substitution of an alkyl amino acid for an alkyl amino acid in SEQ ID NO:4, (2) the substitution of an aromatic amino acid for an aromatic amino acid in SEQ ID NO:4, (3) the substitution of a sulfur-containing amino acid for a sulfur-containing amino acid in SEQ ID NO:4, (4) the substitution of a hydroxy-containing amino acid for a hydroxy-containing amino acid in SEQ ID NO:4, (5) the substitution of an acidic amino acid for an acidic amino acid in SEQ ID NO:4, (6) the substitution of a basic amino acid for a basic amino acid in SEQ ID NO:4, and (7) the substitution of a dibasic monocarboxylic amino acid for a dibasic monocarboxylic amino acid in SEQ ID NO:4.

18. A method for screening compounds that specifically bind with a bHLH-PAS/JHR polypeptide, comprising:

(a) incubating a test compound in a solution that comprises an isolated bHLH-PAS polypeptide, wherein said polypeptide is encoded by the polynucleotide of claim 1, and

(b) detecting the binding of said test compound with said polypeptide.

19. A method for screening compounds that specifically bind with a complex comprising a bHLH-PAS polypeptide that is involved in binding juvenile hormone III and a heteromultimeric partner of said polypeptide, comprising:

(a) incubating a test compound in a solution that comprises an isolated bHLH-PAS polypeptide, and an isolated heteromultimeric partner of said polypeptide, wherein said bHLH-PAS polypeptide is encoded by the polynucleotide of claim 1, and

(b) detecting the binding of said test compound with said complex.

20. The method of claim 18, wherein said test compound is detectably labeled.

21. The method of claim 20, wherein the binding of said test compound with said polypeptide is detected in step (b) using a scintillation proximity assay.

22. The method of claim 20, wherein said detectably labeled test compound comprises a detectable label selected from the group consisting of radiolabel, fluorescent label, chemiluminescent label, and bioluminescent label.

23. The method of claim 18, further comprising the step of incubating said bHLH-PAS polypeptide with a detectably labeled ligand, wherein said detectably labeled ligand is added to said solution containing said receptor at a time selected from the group consisting of (i) prior to step (a), (ii) after step (a) and before step (b), and (iii) concomitantly with the addition of said test compound.

24. The method of claim 18, wherein said detectably labeled ligand is juvenile hormone or a juvenile hormone analog, and wherein said detectable label is selected from the group consisting of radiolabel, fluorescent label, chemiluminescent label, and bioluminescent label.

25. The method of claim 24, wherein said detectably labeled juvenile hormone is [^{3}H]10*R*-juvenile hormone III.

26. The method of claim 24, wherein said detectably labeled juvenile hormone analog is [^{3}H]methoprene.

27. The method of claim 18, further comprising the step of incubating said bHLH-PAS polypeptide with a detectably labeled photoaffinity analog of juvenile hormone after step (a) and before step (b).

28. The method of claim 18, wherein said bHLH-PAS polypeptide is selected from the group consisting of:

(a) a conservative amino acid variant of SEQ ID NO:3,

(b) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:3,

(c) a polypeptide having an amino acid sequence of SEQ ID NO:3,

(d) a conservative amino acid variant of SEQ ID NO:4,

(e) a functional fragment of a polypeptide having the amino acid sequence of SEQ ID NO:4,

(f) a polypeptide having an amino acid sequence of SEQ ID NO:4, and

(g) a *Met*-JHR alternatively-spliced isoform.

29. A nucleic acid probe for detecting RFLPs in an insect population, wherein said RFLPs discriminate between JH-sensitive and JH-resistant individuals, said

probe comprising a genetic locus in a gene encoding a bHLH-PAS polypeptide that is associated with JH analog sensitivity and resistance traits, and wherein said polypeptide is involved in binding juvenile hormone III.

30. A method for detecting JH-resistant individuals in an insect population, said method comprising:

(a) obtaining a representative biological sample of said population; and

(b) detecting a nucleic acid sequence in said sample that corresponds to a predetermined sequence within a polynucleotide encoding a bHLH-PAS polypeptide that is altered in JH analog-resistant individuals, wherein said polypeptide is involved in binding juvenile hormone III.

31. A method according to claim 30, wherein said detecting step comprises:

(i) amplifying a nucleic acid sequence from said sample, wherein said sequence corresponds to a predetermined sequence within a polynucleotide encoding a bHLH-PAS/JHR polypeptide and wherein said sequence comprises at least one RFLP characteristic of JH analog resistance;

(ii) incubating said amplified nucleic acid with at least one predetermined restriction endonuclease, to form fragments;

(iii) size-separating said fragments to form a detectable pattern; and

(iv) comparing said pattern with a predetermined pattern obtained from JH analog-resistant individuals to detect the appearance of one or more RFLP characteristic of JH analog resistance.

32. An *in vivo* method for screening compounds that specifically bind with a bHLH-PAS polypeptide that is involved in binding juvenile hormone III, comprising:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising said bHLH-PAS polypeptide and a second polypeptide comprising a DNA binding domain, and (2) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said bHLH-PAS polypeptide by monitoring expression of the reporter gene.

33. An *in vivo* method for screening compounds that specifically bind with a bHLH-PAS polypeptide that is involved in binding juvenile hormone III, comprising the steps of:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising said bHLH-PAS polypeptide and a second polypeptide comprising a DNA binding domain; (2) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide; and (3) DNA encoding a polypeptide that is a heterodimeric partner of said bHLH-PAS polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said bHLH-PAS polypeptide by monitoring expression of the reporter gene.

34. An *in vivo* method for screening compounds that specifically bind to a multimeric complex comprising a bHLH-PAS polypeptide that is involved in binding juvenile hormone III and the heteromultimeric partner of said polypeptide, comprising the steps of:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising said bHLH-PAS polypeptide

and the DNA binding domain of a second polypeptide, (2) DNA encoding a heteromultimeric partner of said bHLH-PAS polypeptide and the activation domain of said second polypeptide, and (3) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said complex by monitoring expression of the reporter gene.

35. An *in vivo* method for screening compounds that specifically bind to a multimeric complex comprising a bHLH-PAS polypeptide that is involved in binding juvenile hormone III and the heteromultimeric partner of said polypeptide, comprising the steps of:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising bHLH-PAS polypeptide and the activation domain of a second polypeptide, (2) DNA encoding a heteromultimeric partner of said bHLH-PAS polypeptide and the DNA binding domain of said second polypeptide, and (3) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound to said complex by monitoring expression of the reporter gene.

36. An *in vivo* method for screening compounds that specifically bind with a bHLH-PAS polypeptide that is involved in binding juvenile hormone III, comprising:

(a) providing a host cell comprising (1) DNA encoding a fusion polypeptide comprising a bHLH-PAS polypeptide

and the DNA binding region of a second polypeptide, (2) DNA encoding a bHLH-PAS polypeptide and the activation domain of said second polypeptide, and (3) a reporter gene under the control of a minimal promoter driven by the response element for said second polypeptide;

(b) incubating a test compound with said host cell; and

(c) detecting the binding of the test compound with said bHLH-PAS polypeptide by monitoring expression of the reporter gene.

37. A method according to claim 32, wherein said host cell is selected from the group of an insect cell, a yeast cell, and a mammalian cell.

38. A method according to claim 33, wherein said host cell is selected from the group of an insect cell, a yeast cell, and a mammalian cell.

39. A method according to claim 34, wherein said host cell is selected from the group of an insect cell, a yeast cell, and a mammalian cell.

40. A method according to claim 35, wherein said host cell is selected from the group of an insect cell, a yeast cell, and a mammalian cell.

41. A method according to claim 36, wherein said host cell is selected from the group of an insect cell, a yeast cell, and a mammalian cell.

42. An isolated polynucleotide which comprises the sequence of SEQ ID NO:6.

43. An isolated polynucleotide which comprises the sequence of SEQ ID NO:7.

44. An isolated polynucleotide which comprises the sequence of nucleotide 1 through nucleotide 1291 of SEQ ID NO:7.

45. An isolated polynucleotide which comprises the sequence of nucleotide 1 through nucleotide 1513 of SEQ ID NO:7.

46. An isolated polynucleotide which comprises the sequence of nucleotide 3733 through nucleotide 6235 of SEQ ID NO:7.

47. An isolated polynucleotide which comprises the sequence of nucleotide 4302 through nucleotide 6235 of SEQ ID NO:7.

48. An isolated polynucleotide comprising the nucleotide sequence of the St-H fragment in vector pSt-H.

49. The vector pSt-H.

50. An isolated polynucleotide comprising the nucleotide sequence of SEQ ID NO:7.

51. An isolated polynucleotide as claimed in claim 1, wherein said polynucleotide hybridizes under stringent conditions with a polynucleotide having a nucleotide sequence of SEQ ID NO:7.

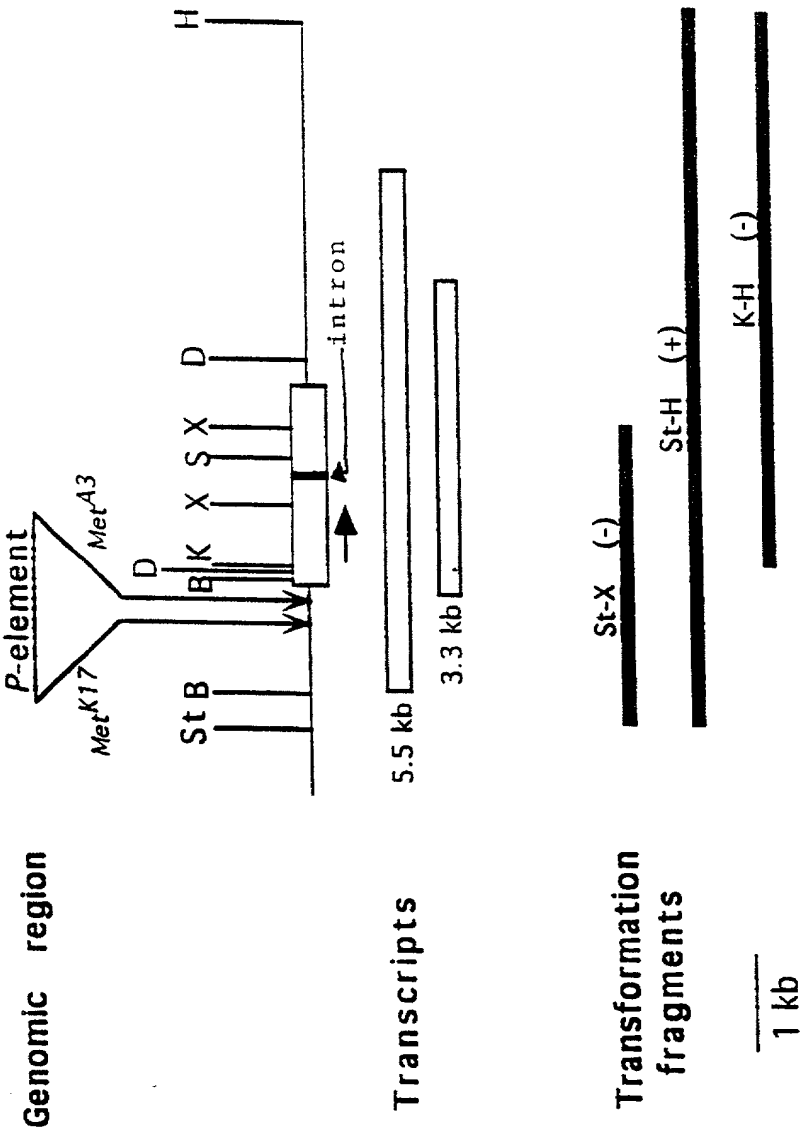

Genomic region
P-element
Met^K17
Met^A3
St B
D
B K X S X D
H
intron
Transcripts
5.5 kb
3.3 kb
Transformation fragments
St-X (-)
St-H (+)
K-H (-)
1 kb

FIGURE 1

6.234kbGENOMIC

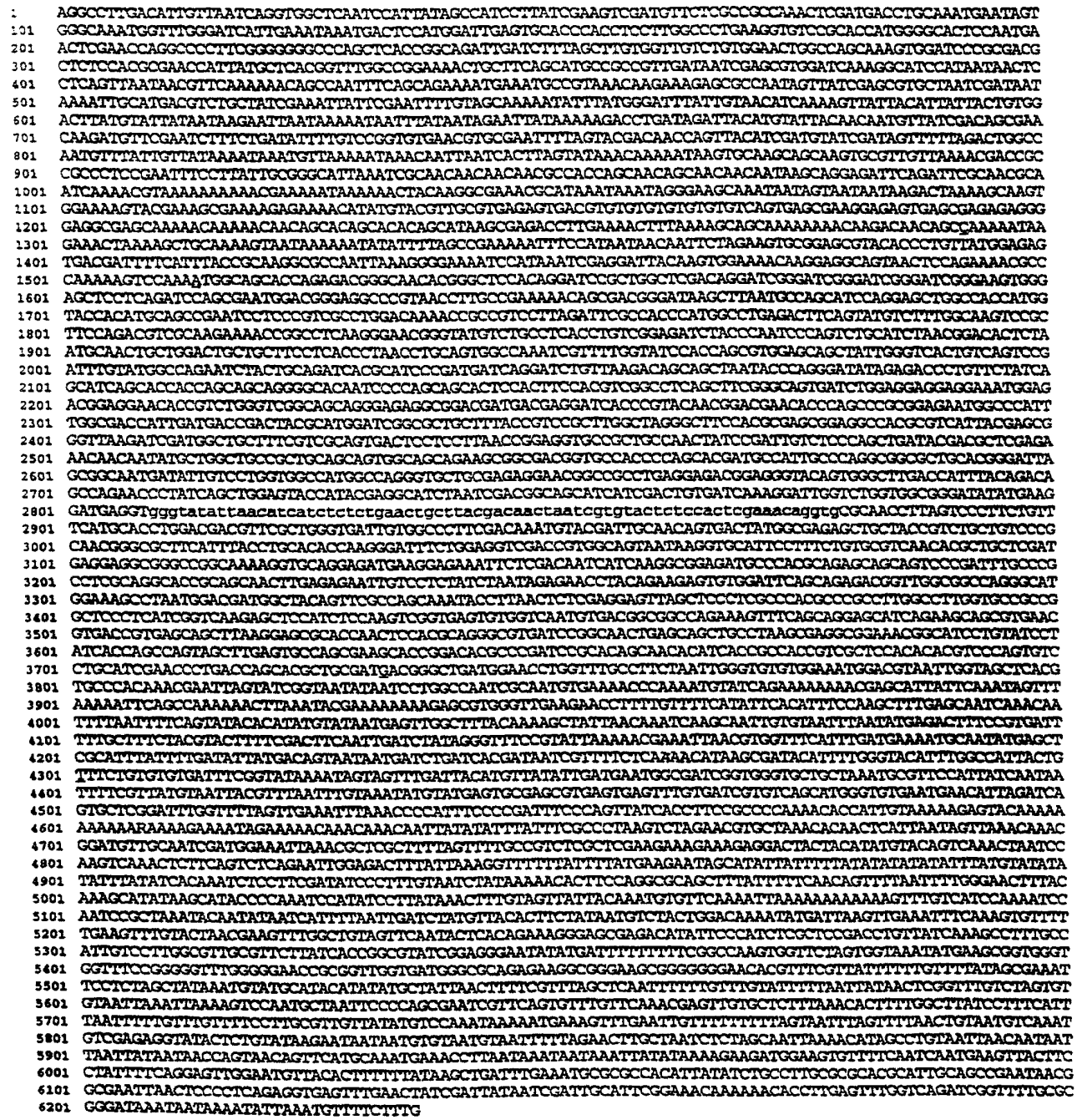


FIGURE 2

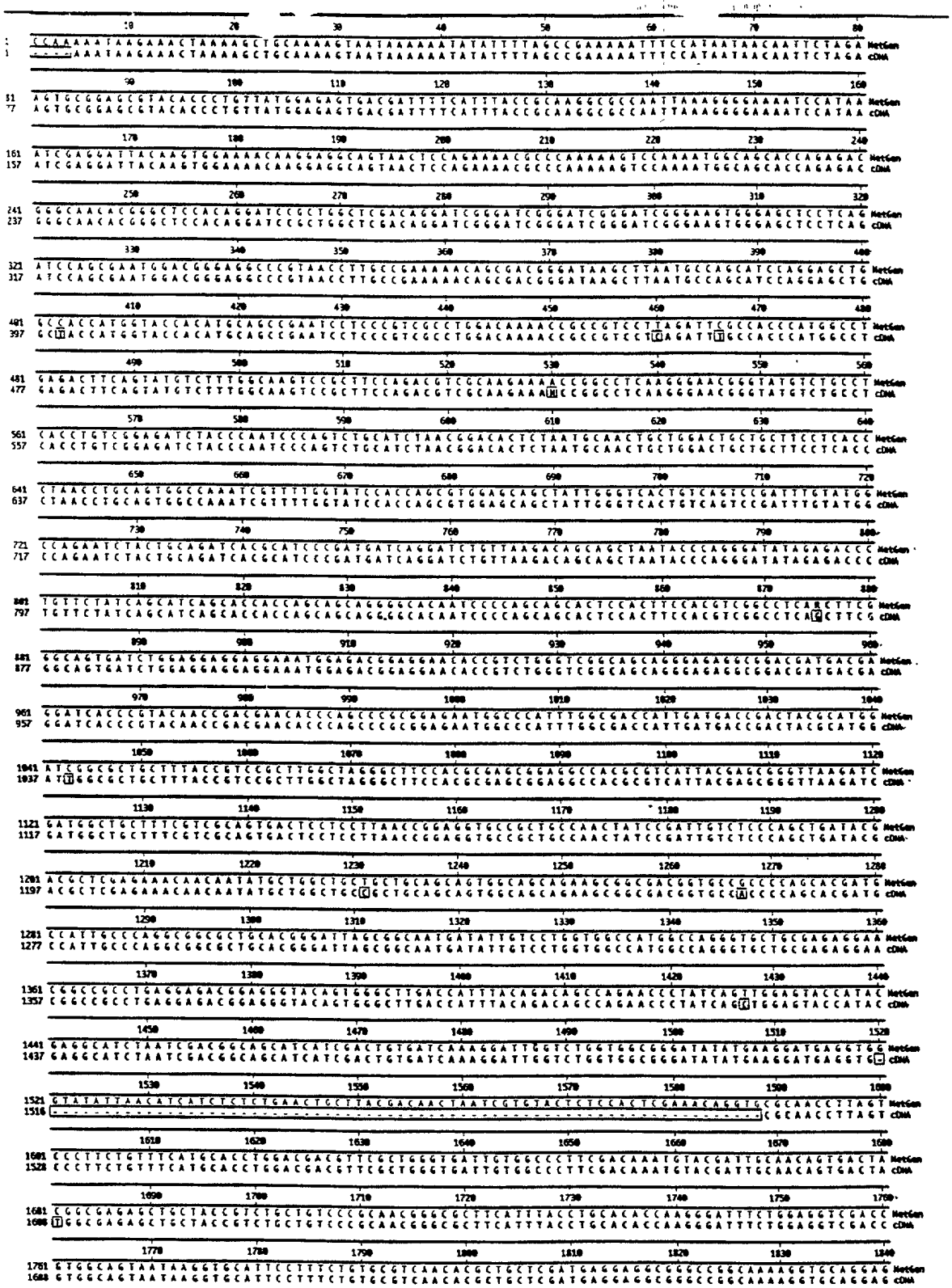


FIGURE 3A

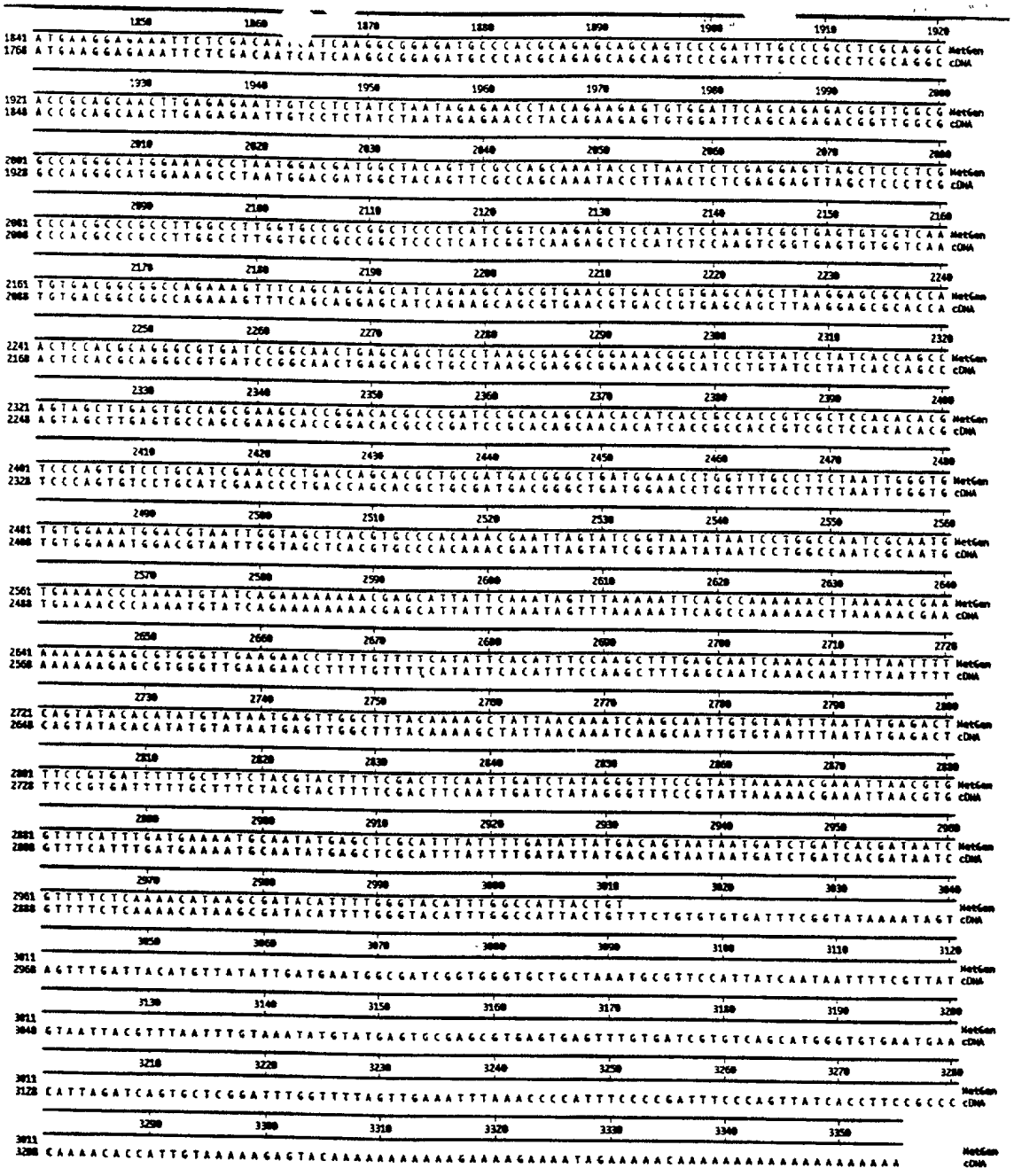
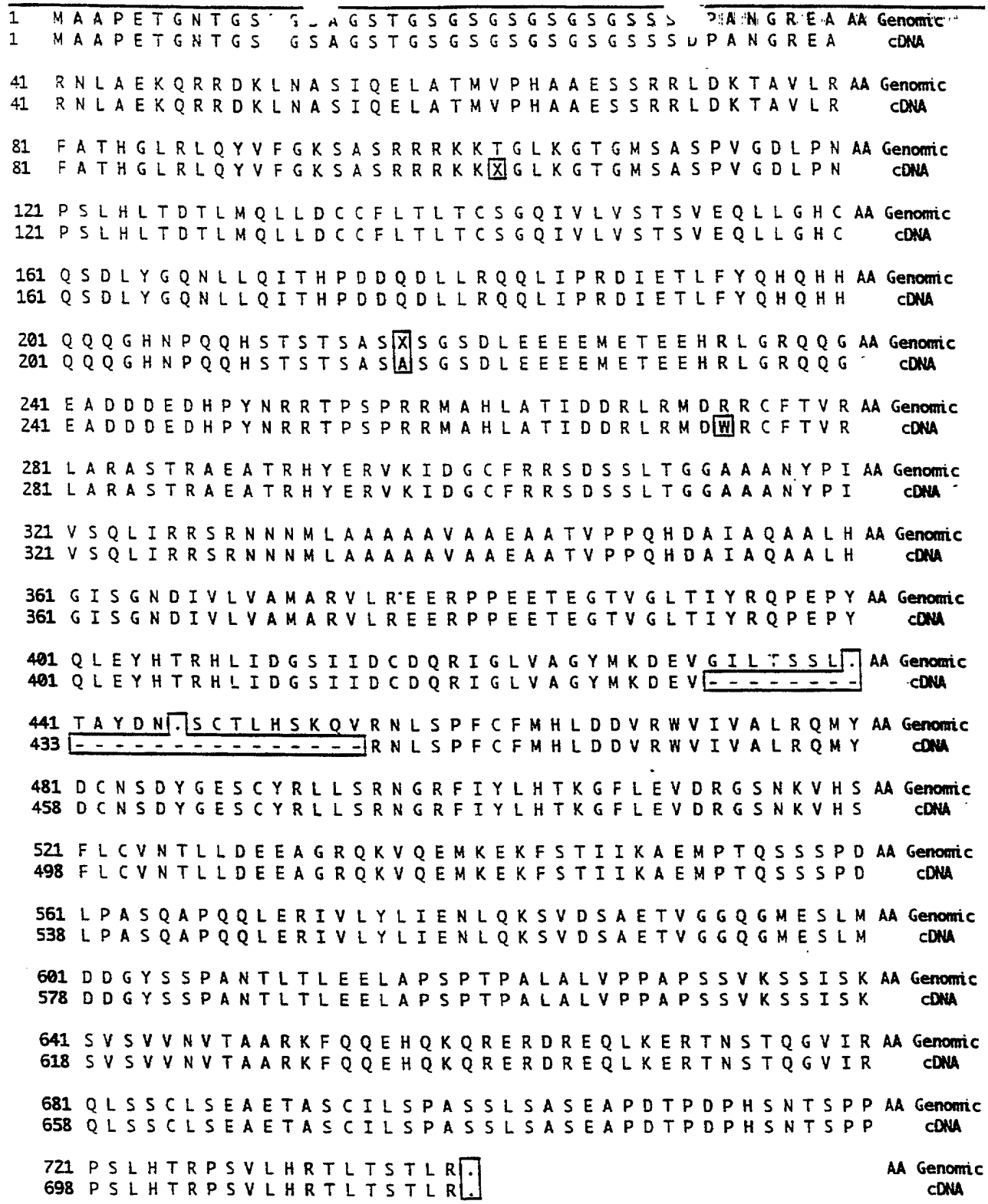


FIGURE 3B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41 R N L A E K Q R R D K L N A S I Q E L A T M V P H A A E S S R R L D K T A V L R AA Genomic
41 R N L A E K Q R R D K L N A S I Q E L A T M V P H A A E S S R R L D K T A V L R cDNA
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81 F A T H G L R L Q Y V F G K S A S R R R K K X G L K G T G M S A S P V G D L P N cDNA
121 P S L H L T D T L M Q L L D C C F L T L T C S G Q I V L V S T S V E Q L L G H C AA Genomic
121 P S L H L T D T L M Q L L D C C F L T L T C S G Q I V L V S T S V E Q L L G H C cDNA
161 Q S D L Y G Q N L L Q I T H P D D Q D L L R Q Q L I P R D I E T L F Y Q H Q H H AA Genomic
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281 L A R A S T R A E A T R H Y E R V K I D G C F R R S D S S L T G G A A A N Y P I cDNA
321 V S Q L I R R S R N N N M L A A A A A V A A E A A T V P P Q H D A I A Q A A L H AA Genomic
321 V S Q L I R R S R N N N M L A A A A A V A A E A A T V P P Q H D A I A Q A A L H cDNA
361 G I S G N D I V L V A M A R V L R E E R P P E E T E G T V G L T I Y R Q P E P Y AA Genomic
361 G I S G N D I V L V A M A R V L R E E R P P E E T E G T V G L T I Y R Q P E P Y cDNA
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441 T A Y D N . S C T L H S K Q V R N L S P F C F M H L D D V R W V I V A L R Q M Y AA Genomic
433 - - - - - - - - - - - - - - - - - R N L S P F C F M H L D D V R W V I V A L R Q M Y cDNA
481 D C N S D Y G E S C Y R L L S R N G R F I Y L H T K G F L E V D R G S N K V H S AA Genomic
458 D C N S D Y G E S C Y R L L S R N G R F I Y L H T K G F L E V D R G S N K V H S cDNA
521 F L C V N T L L D E E A G R Q K V Q E M K E K F S T I I K A E M P T Q S S S P D AA Genomic
498 F L C V N T L L D E E A G R Q K V Q E M K E K F S T I I K A E M P T Q S S S P D cDNA
561 L P A S Q A P Q Q L E R I V L Y L I E N L Q K S V D S A E T V G G Q G M E S L M AA Genomic
538 L P A S Q A P Q Q L E R I V L Y L I E N L Q K S V D S A E T V G G Q G M E S L M cDNA
601 D D G Y S S P A N T L T L E E L A P S P T P A L A L V P P A P S S V K S S I S K AA Genomic
578 D D G Y S S P A N T L T L E E L A P S P T P A L A L V P P A P S S V K S S I S K cDNA
641 S V S V V N V T A A R K F Q Q E H Q K Q R E R D R E Q L K E R T N S T Q G V I R AA Genomic
618 S V S V V N V T A A R K F Q Q E H Q K Q R E R D R E Q L K E R T N S T Q G V I R cDNA
681 Q L S S C L S E A E T A S C I L S P A S S L S A S E A P D T P D P H S N T S P P AA Genomic
658 Q L S S C L S E A E T A S C I L S P A S S L S A S E A P D T P D P H S N T S P P cDNA
721 P S L H T R P S V L H R T L T S T L R . AA Genomic
698 P S L H T R P S V L H R T L T S T L R . cDNA

FIGURE 4

```
1   ATGGCAGCACCAGAGACGGGCAACACGGGCTCCACAGGATCCGCTGGCTCGACAGGATCGGGATCGGGATCGGGAAGTGGGAGC  A

1          CAGCAGACGCGGGCAACACGGGCACCACAGGATCAGCTGGGTCCACA----------GGATCGGGATCGGGAACTGGGACG  P

91  TCCTCAGATCCAGCGAATGGACGGGAGGCCCGTAACCTTGCCGAAAAACAGCGACGGGATAAGCTTAATGCCAGCATCCAGGAGCT  A

72  TCCGCAGATCCAGCGAATGGACGGGAGGCCCGCAATCTTGCCGAGAAACAGCGACGGGATAAGCTTAATGCCAGCATCCAGGAGCT  B

181 GGCTACCATGGTACCACATG-CAGCCGAATCCTCCCGTCGCCTGGACAAAACCGCCGTCCTTAGATTCGCCACCC  A

158 GGCTACCATGGTACCACATGTCAGCCGAATCCTCCCGACGCCTGGACAAAACCGCCGTCCTCAGATTCGCCACCC  B
```

FIGURE 5

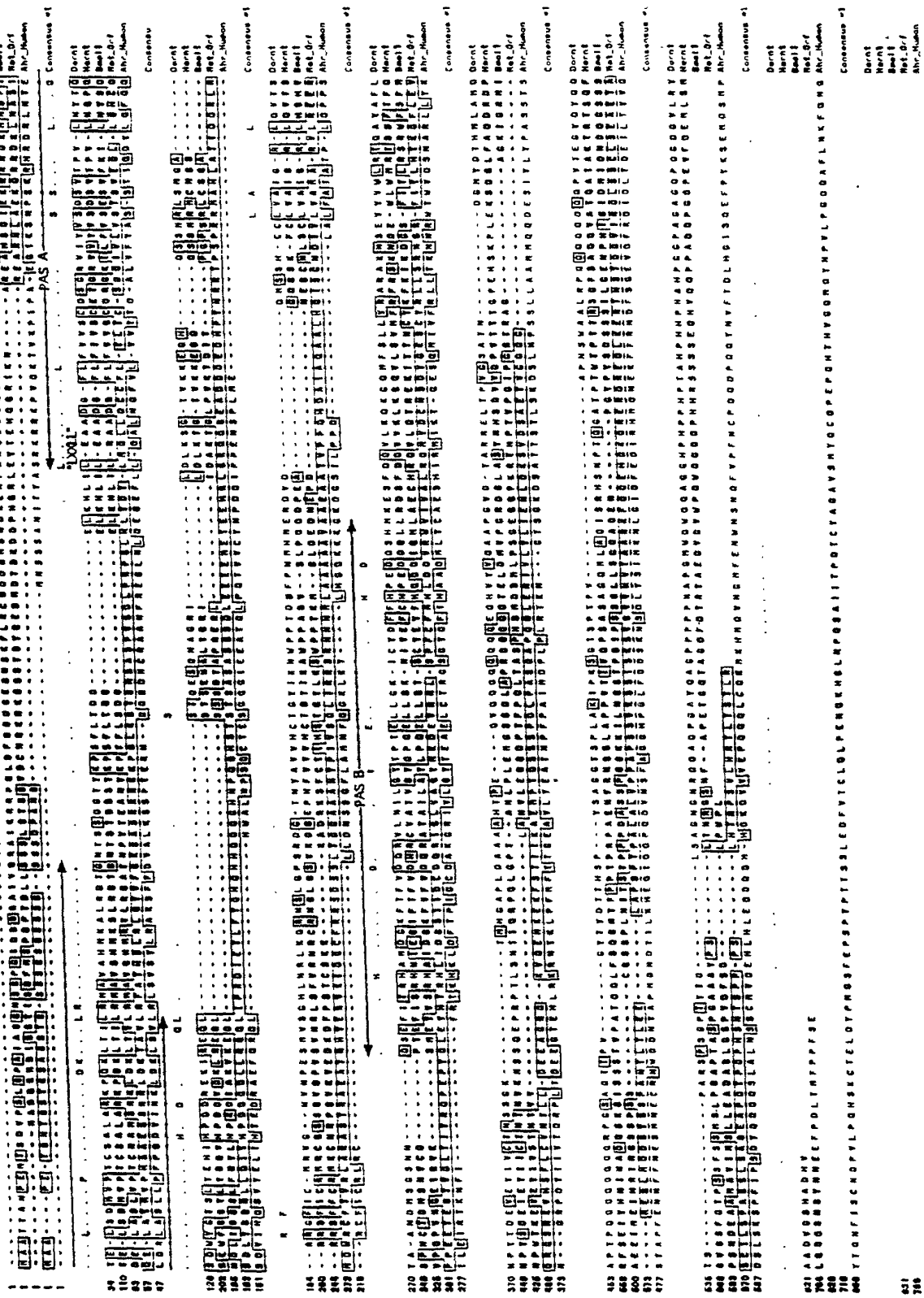

FIGURE 6

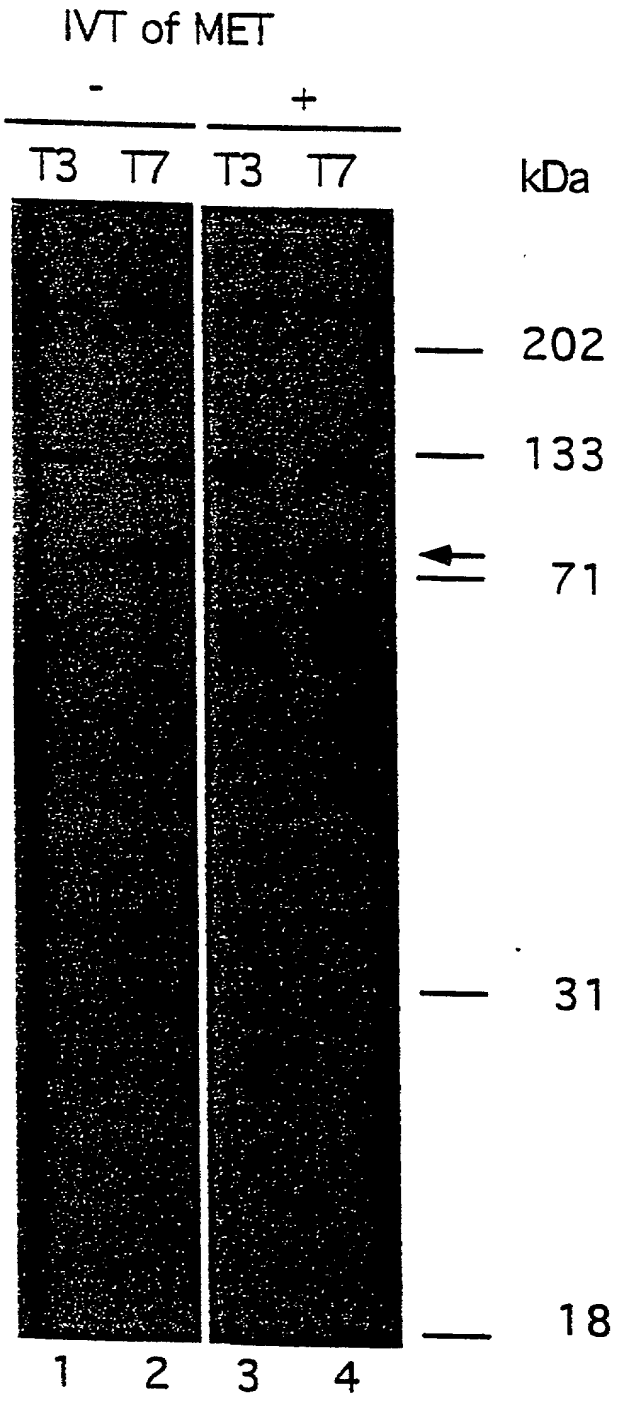

FIGURE 7

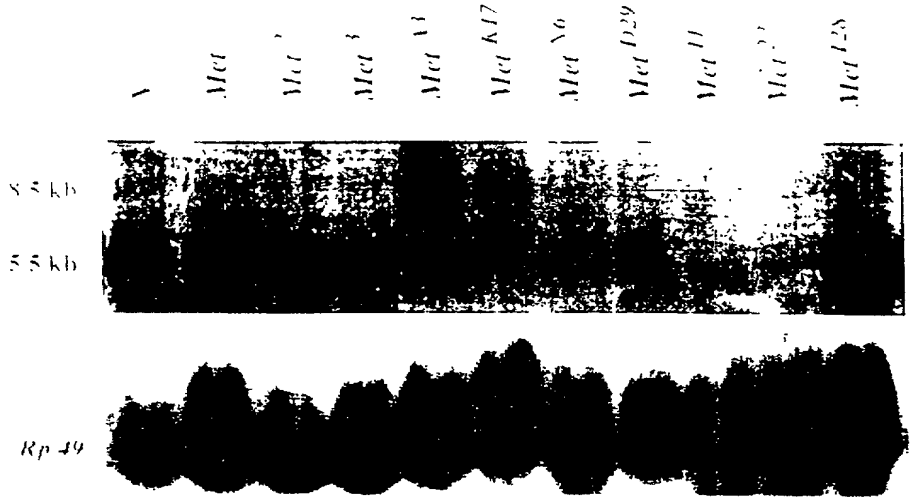

FIGURE 8

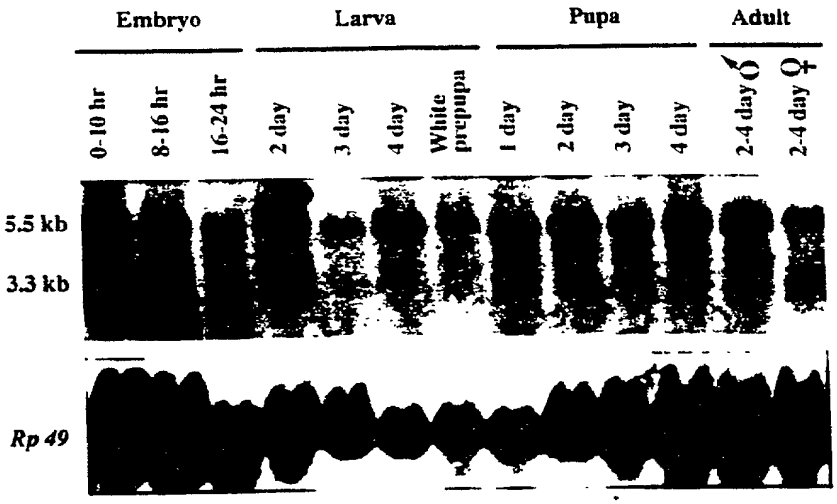

FIGURE 9

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

RECOMBINANT bHLH-PAS/JHR POLYPEPTIDE AND ITS USE TO SCREEN POTENTIAL INSECTICIDES

the specification of which is attached hereto unless the following box is checked:

☒ was filed on *April 14, 1998* as United States Application Number or PCT International Application Number *PCT/US98/07388* and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	PRIORITY CLAIMED
08/843,205	*U.S.A.*	*14/April/1997*	*Yes*

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

APPLICATION NO.	FILING DATE

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is known by me to be material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS: PATENTED, PENDING, ABANDONED
08/971,188	*November 17, 1997*	*PENDING*

I hereby appoint as my attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Stephen A. Bent, Reg. No. 29,768; David A. Blumenthal, Reg. No. 26,257; Alan I. Cantor, Reg. No. 28,163; William T. Ellis, Reg. No. 26,874; John J. Feldhaus, Reg. No. 28,822; Patricia D. Granados, Reg. No. 33,683; John P. Isaacson, Reg. No. 33,715; Michael D. Kaminski, Reg. No. 32,904; Kenneth E. Krosin, Reg. No. 25,735; Glenn Law, Reg No. 34,371; Eugene M. Lee, Reg. No. 32,039; Richard Linn, Reg. No. 25,144; Peter G. Mack, Reg. No. 26,001; Brian J. McNamara, Reg. No. 32,789; Sybil Meloy, Reg. No. 27,789; Richard C. Peet, Reg. No. 35,792; George H. Quillin, Reg. No. 32,792; Colin G. Sandercock, Reg. No. 31,298; Bernhard D. Saxe, Reg. No. 28,665; Charles F. Schill, Reg. No. 27,590; Richard L. Schwaab, Reg. No. 25,479; Arthur Schwartz, Reg. No. 22,115; Harold C. Wegner, Reg. No. 25,258.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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